On the composition and function of the gut microbiome of two insect species, the generalist *Spodoptera littoralis* and the specialist *Melolontha hippocastani*

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Abbreviations and symbols

AMP	antimicrobial peptide
APSE	*Acyrthosiphon pisum* secondary endosymbiont
BLAST	Basic Local Alignment Search Tool
cDNA	complementary DNA
CoA	coenzyme A
CPM	counts per million
dd-H_2O	double-distilled water
DE	differentially expressed
DEPC	diethyl pyrocarbonate
DUOX	dual oxidase
FACS	fluorescence-activated cell sorting
FDR	false discovery rate
FISH	fluorescence *in situ* hybridization
GC-MS	gas chromatography – mass spectrometry
GFP	green fluorescent protein
HDE	highly and differentially expressed
IRMS	isotope ratio mass spectrometry
KEGG	Kyoto Encyclopedia of Genes and Genomes
NCBI	National Center for Biotechnology Information
NSTI	nearest sequenced taxon index
OTU	operational taxonomic unit
PBS	phosphate-buffered saline
PHB	polyhydroxybutyrate

PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PP	pyrophosphate
qPCR	quantitative polymerase chain reaction
rDNA	ribosomal DNA
RNAseq	RNA sequencing
ROS	reactive oxygen species
RPKM	reads per kilobase per million mapped reads
rRNA	ribosomal RNA
SIP	stable isotope probing
TCA	tricarboxylic acid
TEM	transmission electron microscopy
TMM	trimmed mean of M-values

1. General introduction

Insects are the largest class of animals on Earth. Of all the plants and animal species described till date, insects occupy approximately 60% of it. [1]. They can be found in water, on land, in almost any latitude, even southern of the Antarctic circle [2]. Such an extreme widespread distribution implies that insects must embrace a variety of habitats and life styles, which would not be possible without a corresponding physiological adaptation. Concretely, the gut is of particular interest, as this organ is responsible for the processing of the food as well as for excretion of digestive waste. The vast range of diets consumed by the class Insecta is reflected in the diversification of gut structures among different insect species [3]. Also, the digestive tract harbors symbiotic microorganisms, without which insects possibly would not have achieved their ecological success. These partners (microbiome) belong to a wide range of taxonomic affiliations spanning through several domains, encompassing fungi, bacteria, viruses and protozoa [4], [5], [6], [7]. Diet and host taxon are the two most significant factors that influence the composition of the symbiotic community [8], [9].

1.1 General structure and physiochemical conditions of insect guts

The insect gut is a continuous tube divided into three main sections: the foregut, the midgut, and the hindgut (Fig. 1A). The most proximal one, the foregut, is of ectodermal origin. Thus, its cells secrete a chitinous cuticle, known as intima, which is continuous with that covering the outside of the body [10]. The foregut is not involved either in secretion of digestive enzymes or absorption of nutrients. Its main functions are pushing the ingested material towards the midgut and its storage, principally in a subsection called crop [3]. The following section, the midgut, is of endodermal origin and its cells do not secrete cuticle but a more fragile membrane named the peritrophic matrix. Since it is in this region of the digestive tract where most digestive processes take place, the midgut cells actively secrete digestive enzymes into the lumen. Also, the epithelium is covered with microvilli which optimize absorption by increasing the area of contact of the cells with the ingested material up to two orders of magnitude [10]. Usually, the microvilli are covered by a layer of filamentous glycoproteins called glycocalyx, lining the so called ectoperitrophic space, delimited by the other side by the above mentioned peritrophic matrix. In some cases, this membrane packages the food bolus as it moves thought the digestive tract. Either way, the peritrophic matrix is composed of a number of laminae made of a network of

chitin, proteins and glycoproteins. Its main purpose is the separation of the food material from the midgut epithelium, thus protecting the microvilli from abrasion and rendering possible the compartmentalization of enzymatic activity in the endo- and ectoperitrophic spaces [10]. To some extent, the peritrophic matrix also provides protection against harmful chemicals and pathogenic microorganisms, as the pores formed by the proteoglycans (less than 100 nm in diameter) are too small to permit the passage of bacteria [3], [10]. Thus, insects harboring symbiotic bacteria in the midgut confine them to the endoperitrophic space. After the midgut lies the pylorus, sometimes forming a valve between the midgut and the hindgut. Arising from it, the Malpighian tubules collect wastes of different nature from the hemolymph, such as uric acid or alkaloids, and release them to the anterior hindgut [10].

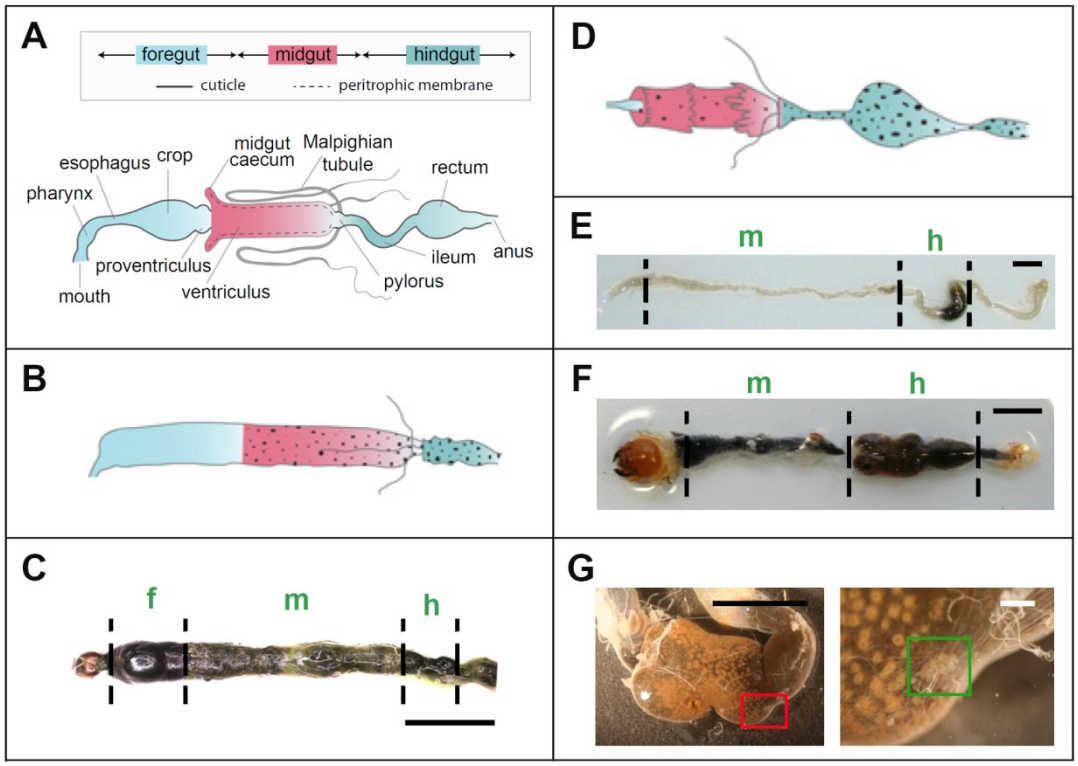

Figure 1. The insect gut. (A) Representation of the general design of the insect gut. (B) Representation of the lepidopteran gut. (C) Gut of a sixth-instar larva of *Spodoptera littoralis*. (D) Representation of the gut of a scarabaeid larvae (Coleoptera). (E) Gut of *Melolontha hippocastani* adult beetle. (F) Gut of third-instar larva of *Melolontha hippocastani*. (G) (left) Close-up of *Melolontha hippocastani*'s larval hindgut. Area within the red square is enlarged on the right. (Right) Detail of *Melolontha hippocastani*'s larval pockets (within the green square). Different colors mark different gut sections in gut schemes. Dashed lines mark different gut section in gut pictures. (f) foregut, (m) midgut, (h) hindgut. Scale bars: black 5 mm, white 0.2 mm. Sources: A, B, D) [3]. C) [193]. E,F,G) This thesis.

Like the foregut, the hindgut is of ectodermal origin, thus, is also covered by the intima layer. This is, however, two orders of magnitude more permeable to molecules up to a certain size than that of the foregut. Polysaccharides and other large molecules cannot diffuse through the hindgut intima. Due to the delivery of waste compounds by the Malpighian tubules, the hindgut internal environment differs from that of the midgut. It contains both food and insect metabolic wastes which are readily available for the symbiotic microorganisms harbored in the pouch (in termites) or the fermentation chamber (in scarabaeids). Regardless of the distinct terminology, these compartments are both an expansion of the hindgut subregion called ileum [10]. The symbiotic microorganisms can thrive either free living or attached to the cuticular spines that line the epithelium of this enlarged section, and the nutrients that they produce (fatty acids from polysaccharide fermentation or amino acids from nitrogenous waste) can be absorbed by the insect [11]. Water from feces is also actively absorbed in the hindgut subregion called rectum, located after the ileum, thanks to the rectal pads (areas with thicker epithelium covered by an unsclerotized intima) [10].

The physiochemical conditions of the gut vary greatly depending on the insect species and the gut region. In general terms pH tends to be alkaline, diverging from that of the hemolymph, which is neutral. In the foregut, pH highly depends on the diet, while in the midgut and hindgut it is actively regulated, the latter being slightly more acidic than the former. Some lepidopterans show an extreme alkalinity in their midguts, with pHs rising until values of 11-12, presumably to enhance the separation of dietary tannins from the digestible food [12]. This has the counterpart of creating an extremely harsh environment for the symbiotic communities. Others show more moderate values, as those of the scarab larvae *Pachnoda ephippiata*, which range from 8 in the anterior midgut, rising to >10 in the center of the midgut, and dropping to almost 7 in the hindgut [13], probably because of the accumulation of acidic short chain fatty acids produced by the hindgut microbiome. Some higher termites show wider ranges of pH, achieving even acidic values (5-6) in the foregut, anterior hindgut and rectum [14]. Oxygen concentration depends on the size of the insect and microbial metabolism. Larger digestive tracts are more prone to create anoxic conditions in the center of lumen than smaller ones. Likewise, robust symbiotic communities tend to consume the oxygen that diffuses through the gut epithelium, contributing to establish a steep oxygen gradient and already achieving anoxic conditions as close as less than 100 μm from the gut wall [15], [16].

1.2 Types of symbionts and mechanisms of transmission

Given the uniformity in their dietary requirements [17], it is astonishing how insects manage to prosper in such a variety of environments and diets [18]. This fact can be thrilling if insects are treated as independent organisms. However, this is not the case, as virtually all the insects are associated with microbial partners. These symbionts in some cases work synergistically with the host, enhancing its metabolic capabilities and allowing it to feed on nutritionally biased diets. As already mentioned, the composition of these symbiotic communities include members taxonomically affiliated to several domains, bacteria being the most common in phytophagous insects [19]. Hence, this thesis focuses on bacterial symbionts only.

In general terms, symbionts fall into two categories: primary, obligate symbionts and secondary, facultative symbionts. Obligate symbionts are mutualists that occur in insects feeding on imbalanced diets, such as plant sap or wood, and usually have a nutritional function, synthetizing the essential compounds which the diet is devoid of [20]. Often, these symbionts are kept within specialized, enlarged insects cells, named either mycetocytes or bacteriocytes (depending on the author). The mycetocytes can be located in the hemocoel, the fat body, or can be part of the gut epithelium; therefore, the symbionts housed there are not part of the gut microbiota *per se* [21]. The bacterium *Buchnera aphidicola*, associated with aphids, is probably the best studied obligate symbiont. It his confined into mycetomes in the hemocoel, and synthetizes essential amino acids in which the phloem that constitutes the aphid diet is very poor. Aposymbiotic aphids have a severely impaired survival rate and fecundity. This reveals the crucial importance that the bacterium has for the host [22]. Obligate symbionts, however, depend as well on their hosts to survive. The extreme genome reduction as a consequence of up to millions of years of symbiotic life [23] has rendered these bacteria unable to survive without the protection of the insect body [24]. Obligate symbionts are strictly transmitted vertically from mother to offspring, usually by infecting the oocytes or the embryos through various mechanisms [25], [26], [27], [28], or encased in “symbiotic shuttles”, that is, symbiont containing capsules that are deposited by the female under the egg mass [29] (Fig. 2). The lack of horizontal transmission, probably related to their extremely low fitness in the open environment, causes congruency between host and symbiont phylogeny [20], [24], [30].

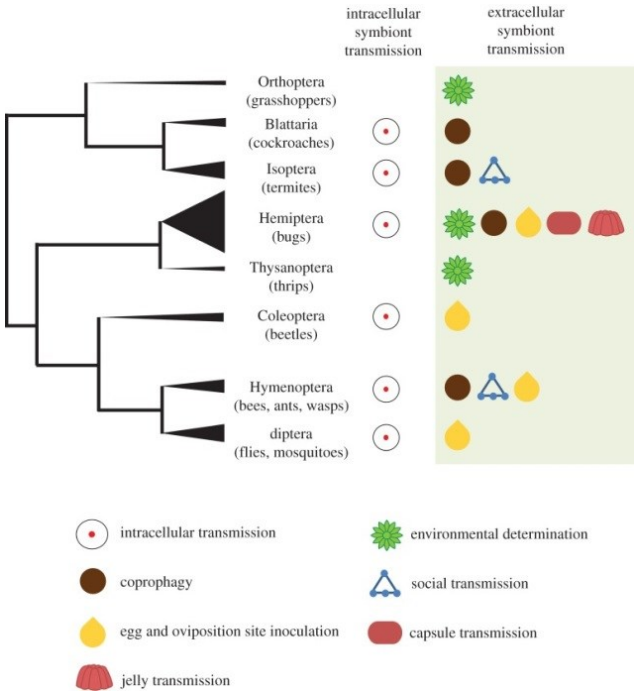

Figure 2. Diversity of insect orders with reported symbiont transmission. Terminal branch thickness is proportional to the number of families within the order that rely on an extracellular route for symbiont transfer. Source: [164]

Facultative symbionts provide less advantages to the insect. Depending on the environmental conditions which the host encounters, the benefits of maintaining a certain facultative symbiont may be higher or lower than its cost. Thus, there is usually an intraspecific variation in whether a particular facultative symbiont is found in an individual [31]. Facultative symbionts are mostly vertically transmitted, although intra- and interspecific horizontal transmission can also occur [32], [33]. Their modes of vertical transmission, although not as stringent as in obligate symbionts, are reliable enough to ensure persistence in the digestive tract across host's generations [34]. Except in few cases [35], facultative symbionts do not invade either germ cells or embryos, but are passed to the offspring through infected "milk secretions" [36], interaction with other members of the colony (in social insects) [37], [38] or by egg surface smearing [39], [40] (Fig. 2). Bacterial transmission from the gut lumen to the inside of the eggs has been described, but to date all cases involve pathogenic and/or transient microorganisms only (immune priming) [41], [42]. The array of roles that the facultative symbionts can undertake is broader than obligate symbionts. Usually, they confer host resistance to pathogens, host plant specificity, even they might favor plant virus transmission by insect vectors [31]. Perhaps the best understood insect – facultative symbiont relationship is the one between the aphid *Acyrthosiphon pisum* and the bacterium *Hamiltonella defensa*. It has been shown that *H. defensa* significantly increases the survival of the aphid upon attack of the parasitoid wasp *Aphidius ervi*

[43]. Interestingly, a third symbiotic partner takes part in this relationship: the lysogenic lambdoid bacteriophage APSE (*A. Pisum* Secondary Endosymbiont). It was noted that the degree of resistance that *H. defensa* confers to the *A. pisum* was depending on genetic variations of the bacteriophage [44]. This case also reveals the double-edged sword that association with facultative symbionts can be: compared to symbiont-free aphids, *A. pisum* associated with *H. defensa* show better fitness in presence of the parasitoid wasp; however, in the absence of the parasitoid, the fitness of the symbiotic aphid is lower than the aposymbiotic one [45].

Many insects do not transmit facultative symbionts from adults to newborn larvae, but they ingest them from the environment along with the diet, leading to significant intraspecific variations in gut community composition. This was first noted in one of the earliest culture-independent study on the gipsy moth [46], which determined that the caterpillars possessed symbiotic communities whose composition was highly dependent on the diet. A similar result was obtained years later in the cabbage white butterfly [47]. In those environmentally acquired symbiosis, the physiochemical conditions of the gut (pH, redox potential, availability of a certain substrate), as well as some phenotypic traits of the bacteria [48], play an essential role in favoring the colonization of the digestive tract by the right bacterial species. The bean bug *Riptortus pedestris*, which depends on an environmental symbiont affiliated within the *Burkholderia* genus [49], has a specialized midgut structure that aid in sorting the beneficial bacterium among the plethora ingested with the diet [50]. In a similar manner, the proventriculus of the desert turtle ant *Cephalotes rohweri* works as a filter that blocks the passage of unwanted bacteria to the midgut [51]. Thanks to all these mechanisms, it is possible to keep the bacterial profiles of the food and the gut well differentiated, even though the former still influences the composition of the latter [3].

1.3 Correlation between location and role of insect symbiotic bacteria

The position of the bacterial symbionts within the insect body greatly determines their contribution to the insect physiology (Fig. 3). For example, obligate symbionts contained in the cytoplasm of mycetocytes usually form long term associations with the host and are better positioned to provide evolutionarily significant capabilities to the insect, such as expansion of host plant range. However, since they are held intracellularly, obligate symbionts are unable to secrete enzymes directly into the alimentary canal; therefore, their participation in digestion or

detoxification of plant compounds is very limited. Moreover, the constrained genetic potential caused by reduced genome size and lack of horizontal gene transfer [52], [53] probably rendered obligate symbionts unfit to provide physiological response to short and long term variations in the environment. Thus, these highly specialized symbionts provide advantages to the insect host only under a narrow range of environmental parameters. On the contrary, facultative symbionts have dynamic genomes, featuring continuous gain and loss of functional genes. Moreover, since they colonize the gut, they are better positioned to actively take part in digestion and detoxification processes. However, their modes of transmission across insect generations may not be reliable enough to provide evolutionarily significant capabilities [54].

It has been shown by both genomic and experimental studies that nutrient provisioning is the cornerstone of obligate symbioses [22], [24], [55]. Available nitrogen is particularly scarce in plants, and herbivore animals typically are nitrogen limited [56]. Therefore, the involvement of obligate symbionts in nitrogen provisioning, mostly in the form of amino acids, it is not surprising. In aphids, the genomes of their symbiotic *Buchnera* spp. have lost up to 90% of original genes but they conserve the pathways for synthesis of essential amino acids, suggesting a role of these symbionts in supplying to the host the amino acids it cannot produce [24], [57]. Experimental data obtained from the aphid-*Buchnera* system is in good agreement with this hypothesis [22], [58]. Concretely, *Buchnera* uses the nonessential amino acids provided by the insect as substrates for the production of the essential ones [55]. A similar mechanism takes place in cockroaches, but in this case the substrate for amino acid synthesis are degradation products of insect's nitrogenous waste. The artificer is the bacterial endosymbiont *Blattabacterium* sp. harbored in mycetocytes associated with the fat body of the insect [59]. This bacterium is able to produce all essential amino acids using urea and ammonia as substrates, enabling cockroaches to thrive in nitrogen-poor decaying wood [60]. In the wood-feeding cerambycid *Tetropium castaneum*, epithelial cells of the proximal midgut serve as housing compartments for bacterial endosymbionts belonging to the *Sodalis* clade [4]. Although the role of this bacterium in nutrition of the cerambycid is not yet clear, the poor nutritional profile of bark-wood based diet makes plausible that it contributes to the sustainment of the insect by providing amino acids, as *Sodalis glossinidius* might do in tsetse flies, based on its genomic profile [61].

Amino acids are not the sole nutrients provided by obligate symbionts. In aphids, *Buchnera* is likely to produce cofactors needed by the host, such as riboflavin, as suggested in a genomic study [57]. Another sap-feeding insect, the sharpshooter *Homalodisca coagulata*, probably relies on its endosymbiont *Baumannia cicadellinicola* for the production of B-vitamins, which are absent in its xylem based diet [62]. Similarly, in insects feeding on blood throughout their whole life cycle, such as cimicids (bed bugs), the anopluran "sucking" lice, *Glossina* flies and other Diptera Pupiparia, mycetocyte-harbored bacterial symbionts are crucial for the supplying of B-vitamins [21]. Also, biosynthetic genes of carotenoids, lipophilic compounds that are lacking in the phloem, remain intact in the genome of *Candidatus* Portiera aleyrodidarum, an endosymbiont of the whitefly *Bemisia tabaci*, despite its drastically reduced genome size [63].

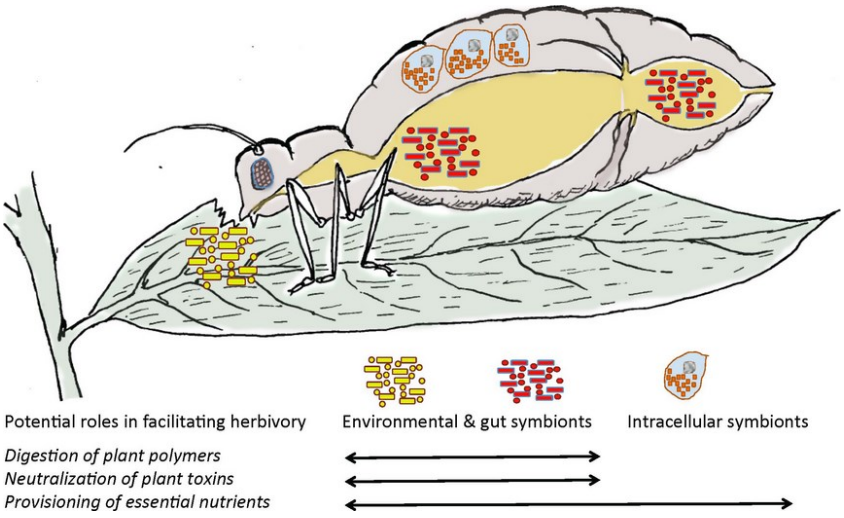

Figure 3. Contribution of symbiotic bacteria to host fitness depends on symbiont location in the body of the insect. Source: [54].

Facultative symbionts, unlike obligate ones, are widespread among insects and virtually all species house at least one facultative symbiotic species [54]. Since they are not kept intracellularly, but either associated with the wall or as free living bacteria in the midgut or hindgut regions, they are in direct contact with the ingested food and better positioned to contribute to the host's digestive machinery. In most heteropteran species, the symbiotic bacteria are harbored not simply within the gut fluid or attached to the wall, but in specialized compartments that ensure their persistency in the digestive tract by keeping them away from the constant influx of ingested food and non-symbiotic microbes. These insects share analogous caeca, or crypts, located at the distal region of their midgut. The crypts contain one single symbiotic phylotype, that varies depending on the host species, although symbiotic monophyly

occurs among recently diverged insect taxa [64], [65]. The stinkbug *Megacopta punctatissima* is associated with a γ-proteobacterium [66], whereas the occupier of the crypts of the stinkbugs *Leptocorisa* sp. and *Riptortus* sp. is a β-proteobacterium belonging to the genus *Burkholderia* [48], [67]. Other insects with singular bacteria-housing organs are *Tetraponera* ants [68].

As stated before, facultative symbionts may take part in a wider variety of roles than obligate symbionts. In particular, their implications in the digestion of recalcitrant compounds such as cellulose have been extensively addressed. Cellulose is a carbon rich polymer, ubiquitous in plants, but since it exists as crystalline or amorphous microfibrils in cell walls, it is not readily digestible by the insect [69]. Moreover, cellulose forms intimate complexes with hemicelluloses and lignin, hindering even more any enzymatic attack [38]. The disruption of such complexes and the cellulose microfibrils into simpler oligosaccharides is crucial in order to render cellulose accessible to degrading enzymes. Gut symbiotic bacteria are typically involved in this process [70]–[72] [73], [74], as well as cellulases of insect origin [75][76]–[79]. However, the relative contribution of insect- and bacteria-derived cellulases in the overall process of cellulose breakdown is not yet clear, and it may fluctuate depending on insect species, gut community structure and diet composition [3]. Termites, due to their ubiquity and impact on human activities, have gained the focus of most of the research on lignocellulose digestion [37], [77], [78]. These insects are often classified into two groups: the lower termites, whose symbiotic community is composed of a complex network of bacteria, protists and archaea, and the higher termites, whose digestive tract is devoid of protists and contain exclusively bacteria and archaea [38]. The bacterial involvement in lignocellulose digestion is more significant in higher than in lower termites, as in the latter, the protist fraction of the gut community carry out part of the process. In higher termites, the taxonomic affiliation of the cellulolytic symbionts is variable across host species. In the best studied case, the wood-feeding *Nasutitermes* spp., the main cellulose degraders are bacteria of the phylum Fibrobacteres [70], [82]. Other cellulolytic bacteria detected in the gut of higher termites are *Bacillus* sp. [83], *Acinetobacter* sp., *Pseudomonas* sp., *Staphylococcus* sp. and Enterobacteriaceae representatives [84].

Scarabaeid larvae can be compared to termites with regard of diet and hindgut morphology (enlarged fermentation chamber). The first report on cellulose digestion by scarabaeid gut bacteria is a study on the larva of the rose chafer *Potosia cuprea* conducted by Werner in 1926,

in which a cellulolytic *Bacillus* sp. was isolated from the hindgut of this insect [85]. Later works highlighted the presence of a strongly alkaline midgut, where lignocellulolytic fibers are fragmented enzymatically, followed by a highly anoxic hindgut where further degradation and microbial fermentation of saccharides takes place [86]–[88]. More recently, a survey on the larvae *Costelytra zealandica* detected β- and *δ*-proteobacteria, Bacteroidetes and Clostridia in the hindgut and suggested *Clostridium* spp. as the main cellulose fermenters [89] and in a culture-based study on the larva of *Holotrichia parallela* a variety of cellulolytic bacteria belonging mainly to the Proteobacteria phylum were isolated from the hindgut [90]. These findings put the scarabaeid symbiotic community in close similarity with that of termites, composed mainly by Proteobacteria, Spirochetes, Bacteroides and Clostridiales [91]–[93]. Among the Proteobacteria, the *Citrobacter* genus is likely to be the most active cellulose degrading in scarabaeids, as it has been isolated from multiple species and its ability to break down lignocellulolytic material has been widely demonstrated [87], [89], [90], [94].

In some insects lacking obligate symbionts and feeding in low-nitrogen diets, extracellular facultative symbionts are involved in supplying this element in an utilizable form for the host. Comparably to *Blattabacterium* sp., *Bacteroides* and *Citrobacter* species of the hindgut of the termite *Reticulitermes flavipes* contribute to nitrogen conservation by allowing its incorporation from waste uric acid into insect tissue [95]. In another study on an array of wood-feeding termite species, different uric acid degrading bacteria were isolated, presumably involved in nitrogen recycling as well. Those belonged to the classes Clostridia and Bacilli and the family Enterobacteriaceae [96]. Nevertheless, microbial conservation of nitrogen is not exclusive of termites. Facultative symbionts of other insects thriving in unbalanced diets also have shown the ability to avoid excessive nitrogen loss. The shield bug *Parastrachia japonensis* is suggested to rely on its midgut symbiont *Erwinia* sp. for the conversion of uric acid into usable amino acids during diapause [97]. In a study on the wood-feeding cerambycid *Anoplophora glabripennis*, the occurrence of a nitrogen recycling mechanism was demonstrated by mixing ^{15}N labeled urea with the diet of the cerambycid, and later on detecting the labeled nitrogen in insect tissue. Although the bacterium behind this phenomenon remains unknown, they hypothesized that it might be an Enterobacteriaceae related species. Additionally, the detection of *nifH* transcripts and a positive acetylene reduction assay suggested that nitrogen fixation occurs in parallel to the recycling of nitrogenous waste [98]. To date, nitrogen fixation as a way of balancing the

overload of dietary carbon has been broadly reported in the alimentary canal of many insect species, and in all cases it is carried out by facultative symbionts. Similar to *A. glabripennis*, gut symbionts of the bark beetles *Dendroctonus* sp. showed this dual ability of taking advantage of both waste and atmospheric nitrogen [99], [100]. Two strains of nitrogen-fixing *Enterobacter agglomerans* were isolated from the gut of the wood-feeding termite *Coptotermes formosanus* [101]. Also Enterobacteriaceae representatives may contribute to the intake of nitrogen of the fruit fly *Ceratitis capitata* by performing nitrogen fixation [102]. In the larvae of the stag beetle *Dorcus (Macrodorcus) rectus* nitrogen fixation was suggested, although the responsible microorganism was not elucidated [103]. Finally, in *Tetraponera* ants, bacterial symbionts closely related to nitrogen-fixing root-nodule bacteria were spotted in specialized pouches close to the Malpighian tubules, although their location suggests that they might not be involved in nitrogen fixation but in recycling of nitrogenous waste [68].

Facultative symbionts also can be engaged in protective roles that increase the fitness of the host under certain conditions. A clear example of that is the already discussed case of the bean bug *Riptortus pedestris* and its environmental β-proteobacterial symbiont *Burkholderia* sp. [49]. It has been shown that when this insect is in contact with the insecticide fenitrothion, *Burkholderia* sp. is capable of degrading it, probably due to the presence of the corresponding gene obtained by horizontal transfer from nearby environmental bacteria [104]. Detoxification of plant secondary metabolites is also a typical function suggested for facultative symbionts. A metagenomic survey of the gut bacterial community of the bark beetle *Dendroctonus ponderosae* unveiled a high occurrence of terpene degradation genes, a common defensive compound of the pines that constitute its diet [105]. Also, studies on the velvet caterpillar *Anticarsia gemmatalis*, which feed on plants rich in protease inhibitors, showed that the survival and growth rates of the caterpillar were higher in individuals with intact gut community, and that gut isolates secreted proteases which were unaffected by the inhibitors produced by the plant [106], [107]. In some cases, facultative symbionts do not protect against defensive molecules, but they can enhance host immunity against certain pathogens, as in the already discussed case of the aphid *Acyrthosiphon pisum* and the bacterium *Hamiltonella defensa*, which protects its host against the wasp *Aphidius ervi* [43], or the defensive mutualist *Enterococcus mundtii* within the gut of the lepidopteran *Spodoptera littoralis* [108], as well as the bacterium *Pantoea agglomerans*, which

produces quinines that inhibit the germination of the pathogenic fungus *Metarhizium anisopliae* in the gut of the desert locust *Schistocerca gregaria* [109].

1.4 *Melolontha hippocastani*: life cycle, gut physiochemical conditions and known bacterial symbionts

One of the subjects of study of this thesis is the forest cockchafer (*Melolontha hippocastani*), a member of the Scarabaeidae family (Fig. 4). *M. hippocastani* is endemic of Euro-Asia and it has great economic importance due to the damage that it infringes to the vegetation during occasional population outbreaks [110]. Its life cycle lasts from 3 to 5 years, depending on the climatic conditions. The development of the larvae takes place underground, where they have access to the roots that constitute their food source. Larvae go through three instars (L1, L2 and L3), after which pupation and metamorphosis take place. Metamorphosis elapses for 2 months and is finished in August, but newly formed adults remain in the pupae case until next April-May in a diapausing state. When the flying season starts, adults emerge from the soil, fly to the canopy of the threes and start feeding on the leaves. After 2 or 3 weeks, the females dig themselves for 3-4 days into a depth of 10-40 cm and lay the eggs next to the roots the future larvae will feed on. An adult female can lay eggs up to two times in the 4-6 weeks of duration of its life span [111].

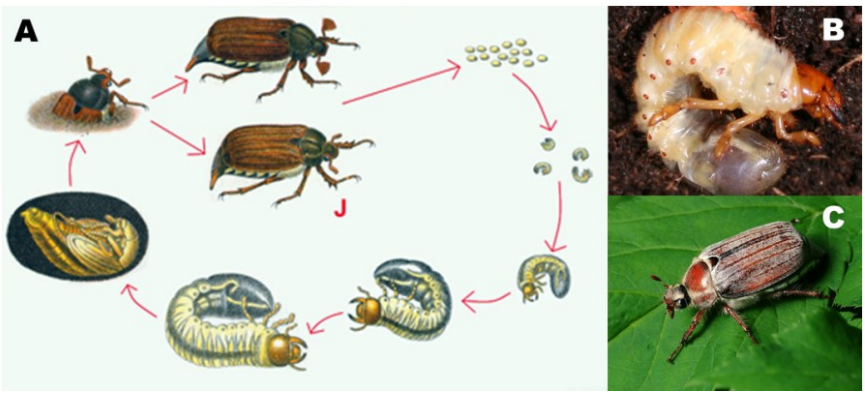

Figure 4. *M. hippocastani*. (A) Overview of the cycle, consisting of 3 larval instars (L1, L2, L3) and adult stage. (B) Third instar larva. (C) Adult beetle. Sources: A) https://commons.wikimedia.org. B) This thesis. C) http://macroid.ru

In *Melolontha* sp. larvae, the digestive tract consist of a negligible foregut, a tubular midgut and an enlarged hindgut (Fig. 1D,F,G); in adults, the midgut is elongated and the hindgut is reduced in volume (Fig. 1E). The pH is somewhat alkaline, and rather constant along the whole length of the tract (from 7.9 in the midgut to 8.6 in the hindgut). Redox potential ranges from +220 to

+340 mV in the midgut, reaches a minimum in the midgut-hindgut junction (0 mV) and increases again towards the rectum. Steep gradient of O_2 is present in the whole gut, with an aerobic-microaerophilic area penetrating less than 100 μm into the lumen, and H_2 accumulates only slightly in the midgut. Short chain fatty acids produced by microbial fermentation, particularly acetate, accumulate in both compartments, principally in the midgut [16]. The bacterial community, composed of facultative symbionts, is more complex and stable in the hindgut, as the midgut is mainly a site of plant polymer degradation and inactivation of dietary microorganisms by insect-produced enzymes [16], [112], [113]. The symbiotic diversity in the hindgut entails phylotypes not only found in termites and cetoniid beetles, but also in ruminants. A study on *Melolontha melolontha* larvae unveiled radial variations in bacterial distribution. In the hindgut lumen, the order Clostridiales and *Turicibacter sanguis* (order Erysipelotrichales) dominated; less frequent were members of Actinobacteria, Bacillales, Lactobacillales, γ-proteobacteria and *Dysgononomonas*-related Bacteroidetes. In the hindgut wall the Clostridiales showed higher relative abundance than in the lumen, followed by *Bacteroides*- and *Chytophaga*-related Bacteroidetes and the genus *Desulfovibrio*, which was not detected in the lumen but showed an abundance of 15% in the wall [16]. Another study on the larval midgut and adult whole gut symbiotic diversity of *Melolontha hippocastani* found that the bacterial community remained constant across the complete life cycle of the insect, regardless of the striking difference between adult and larval life style. This core bacteriome was composed by representatives of γ-, β- and δ-proteobacteria, Bacilli, Clostridia, Erysiphelotrichi and Sphingobacteria classes. However, at lower taxonomic levels, the number of phylotypes decreased significantly in adults compared to larvae, reflecting a simplification of the symbiotic community in later life stages [114].

In addition to the typical scarabaeid gut structure, *M. melolontha* and *M. hippocastani* larvae display uncommon structures at both sides of the distal end of the hindgut (Fig. 1G). These formations, hitherto only once described in the literature, are also present in the rhizophagous grub of the genus *Anomala* [115]. The purpose they serve is, to date, unknown, although their shape suggest analogy to the midgut crypts in Heteroptera [66], [67] or the symbiont-housing organ in the hindgut of *Tetraponera* ants [68].

1.5 *Spodoptera littoralis*: life cycle, gut physiochemical conditions and known bacterial symbionts

The other subject of study of this thesis is the cotton leafworm (*Spodoptera littoralis*), belonging to the family Noctuidae (Fig. 5). This widely distributed lepidopteran is able to feed on a broad range of plants covering more than 40 families, of which around 100 species are of economic relevance [116]. Its life cycle is quite affected by temperature and low humidity. It comprises six larval instars (L1 to L6) which are rapidly completed, altogether in 15-23 days at 25 ^{o}C. During the larval stage, *S. littoralis* voraciously feeds on a single leaf during the first 3-6 days after hatching and afterwards they spread all over the plant. The pupation stage is spent in the soil and lasts 11-13 days at 25 ^{o}C. 2-5 days after adult emergence, females lay 1000-2000 eggs in groups of 25 to 1000 in the adaxial side of host plant leaves, which will hatch in about 4 days in summer or 11-12 in winter [117]. Adult life span is 4-10 days, females living longer than the males [118]. The whole life cycle can be completed in only 5 weeks.

The digestive tract of *S. littoralis* larvae is composed of a long foregut (8 mm in third instar) and midgut (14 mm in third instar) and a relatively short hindgut (4 mm in third instar) (Fig. 1B,C). The pH decreases markedly across the proximal-distal axis, being strongly alkaline in the foregut (around 10) and approaching neutrality towards the anus (midgut pH around 9, hindgut pH around 8, dropping to 7 in the distal hindgut section) [119]. This marked alkalinity, coupled by a rapid food passage through the gut, creates a hostile environment for bacteria. As a result, in *S. littoralis*, as in many other lepidopterans, the gut microbiome is quite simple compared to other insect orders. It lacks of known obligate symbionts. Early-instar larval guts are mainly colonized by Proteobacteria and Firmicutes of the genera *Pantoea*, *Citrobacter* and *Enterococcus*. In late instars, the Proteobacteria are almost completely replaced by Firmicutes of the genera *Clostridium* and *Enterococcus*. This taxonomical shift could possibly be driven by changes in host physiology such as drop in gut redox potential and enhanced immune response, as well as by the growth of the digestive tract which favors the formation of anoxic areas within [120]. Nevertheless, prediction of bacterial metagenome in both early and late larval stages suggested that the symbiotic bacteria might undertake similar roles across the whole larval stage, i.e. plant polysaccharide degradation. Studies involving analysis of bacterial RNA and Stable Isotope Probing revealed that *Enterococcus* sp. is not only the most dominant genus but also the most

metabolically active [108], [120]. Furthermore, its isolation and phylogenetic analysis allowed to place it with the *Enterococcus mundtii* species and revealed its ability to produce antimicrobial peptides, which are possibly used to kill both competitors and entomopathogenic bacteria. Altogether, these data suggest that the gut microbiota in *S. littoralis* may be beneficial to the host in terms of both defense and nutrient provisioning [108].

Figure 5. *S. littoralis*. (A) Fourth instar larva. (B) Adult moth. Source: http://ukmoths.org.uk

1.6 Goals of the thesis

a) In view of the unexpected community stability across the entire life cycle of *M. hippocastani*, we aim to identify, in both larvae and adults, the active microbial fraction involved in two crucial physiological processes for insects feeding on high C/N diets: the degradation of cellulose and the recycling of nitrogen. This question will be addressed using Stable Isotope Probing using ^{13}C labeled cellulose and ^{15}N urea as trophic links coupled with Illumina sequencing (Illumina-SIP).

b) Having previously identified the key bacterium in the gut of *S. littoralis*, *E. mundtii*, by means of Stable Isotope Probing with ^{13}C glucose as trophic link coupled with 454 pyrosequencing (Pyro-SIP), we expect to go deeper into the function of this symbiotic microbe by sequencing and analyzing its genome.

c) To characterize, by both microscopic and high-throughput sequencing techniques, the bacterial community inhabiting the uncommon structures located at the distal end of the larval hindgut of *M. hippocastani* and compare it to that of the surrounding hindgut wall.

d) To get an insight into the genes expressed in these structures and, therefore, into their function, by determining their transcriptome by means of Illumina HiSeq and compare it to that of the surrounding hindgut wall.

e) To compare the symbiotic communities of a generalist insect (*Spodoptera littoralis*) and a specialist one (*Melolontha hippocastani*).

2. Thesis outline – list of articles with author's contribution

2.1 Article I

In Vivo Isotopic Labeling of Symbiotic Bacteria Involved in Cellulose Degradation and Nitrogen Recycling within the Gut of the Forest Cockchafer (*Melolontha hippocastani*)

Pol Alonso-Pernas[1], Stefan Bartram[1], Erika M. Arias-Cordero[1], Alexey L. Novoselov[1], Lorena Halty-deLeon[2], Yongqi Shao[3] and Wilhelm Boland[1]

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In this article, relevant gut bacteria of *M. hippocastani* was labeled and identified with a combination of Stable Isotope Probing with Illumina MiSeq (Illumina-SIP). Cellulose and urea were used as experimental substrates in order to target bacteria involved in cellulose degradation and nitrogen recycling. Labeled nitrogen was detected in insect tissue with Isotope Ratio Mass Spectrometry (IRMS), confirming reincorporation of waste nitrogen to insect body. ^{13}C cellulose labeled bacterial families were Lachnospiraceae and Enterococcaceae in larvae and Enterobacteriaceae in adults. ^{15}N urea labeled bacterial genera were *Burkholderia* in larvae and *Parabacteroides* in adults. This confirms that, although the overall composition of the community is fairly constant during the entire insect life cycle, there is a taxonomic shift in the metabolically active bacteria coupled with the transition from larvae to adults, possibly as a result of the adaptation to the new lifestyle.

Designed the experiments: PA, EA, AN, YS. Performed the experiments: PA, SB. Analyzed the data: PA, AN, LH. Wrote the manuscript: PA, AN. Conceived and supervised the project: WB.

2.2 Article II

Draft Genome Sequence of *Enterococcus mundtii* SL 16, an Indigenous gut Bacterium of the Polyphagous Pest *Spodoptera littoralis*

Bosheng Chen[1*], Chao Sun[2*], Xili Liang[1], Xingmeng Lu[1], Qikang Gao[2], Pol Alonso-Pernas[3], Beng-Soon Teh[3], Alexey L. Novoselov[3], Wilhelm Boland[3] and Yongqi Shao[1]

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In this paper, the genome of *E. mundtii* strain SL 16, a major symbiotic bacterium of the gut of the polyphagous lepidopteran *S. littoralis*, was sequenced in order to get insight on the function of this bacterium within the gut and provide a reference genome for future research. The sequence revealed a high amount of genes devoted to carbohydrate transport and metabolism and suggested the ability of this bacterium to produce fermentative short-chain fatty acids such as formate and acetate. The metabolism of an array of saccharides (cellobiose, xylose, arabinose and sucrose) was tested *in vitro* with positive result. This suggests that this symbiont plays a crucial role in the digestion of diet carbohydrates, thus promoting host development.

Designed the experiments: YS, WB. Isolated the bacterium: XLi, BT. Performed the experiments: YS, WB, BC, CS. Sequenced the DNA: QG, XLu. Performed the bioinformatic analysis: AN, PA. Conceived and supervised the project: XS, WB.

2.3 Article III

Bacterial Community and PHB-Accumulating Bacteria Associated with the Wall and Specialized Niches of the Hindgut of the Forest Cockchafer (*Melolontha hippocastani*)

Pol Alonso-Pernas[1*], Erika Arias-Cordero[1*], Alexey Novoselov[1], Christina Ebert[2], Jürgen Rybak[3], Martin Kaltenpoth[4], Martin Westermann[5], Ute Neugebauer[2] and Wilhelm Boland[1]

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In the present study a survey on the bacterial community of the hindgut wall of *M. hippocastani* was carried out focusing on three life stages (second- and third instar larvae and adults). Furthermore, specialized symbiotic niches attached to the hindgut wall (the pockets), hitherto very poorly described in the literature, were explored in second instar larvae. 454 pyrosequencing revealed that the hindgut wall community varies depending on host life stages, being in third instar larvae similar to that of adults. The community of the pockets was markedly different to that of the hindgut wall. Pocket bacteria accumulated intracellular poly-β-hydroxybutyrate (PHB), as revealed by Transmission Electron Microscopy (TEM) and Raman microspectroscopy. The genus *Achromobacter* (Alcaligenaceae family) dominated the pockets. Gas Chromatography coupled with Mass Spectrometry (GC-MS) revealed that *Achromobacter*

sp. and was able to accumulate PHB *in vitro*. The accumulation of this polymer might play a role in ensuring successful colonization of the pockets.

Designed the experiments: PA, EA, AN. Performed the experiments: PA, EA, AN, CE, JR, MW. Analyzed the data: PA, EA, AN, JR, MK. Wrote the manuscript: PA, EA, AN, MK. Supervised the project: UN, WB.

3. Articles with author's contribution

3.1 Article I

In Vivo Isotopic Labeling of Symbiotic Bacteria Involved in Cellulose Degradation and Nitrogen Recycling within the Gut of the Forest Cockchafer (_Melolontha hippocastani_)

Pol Alonso-Pernas, Stefan Bartram, Erika M. Arias-Cordero, Alexey L. Novoselov, Lorena Halty-deLeon, Yongqi Shao and Wilhelm Boland

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In Vivo Isotopic Labeling of Symbiotic Bacteria Involved in Cellulose Degradation and Nitrogen Recycling within the Gut of the Forest Cockchafer (*Melolontha hippocastani*)

*Pol Alonso-Pernas[1], Stefan Bartram[1], Erika M. Arias-Cordero[1], Alexey L. Novoselov[1], Lorena Halty-deLeon[2], Yongqi Shao[3] and Wilhelm Boland[1]**

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The guts of insects harbor symbiotic bacterial communities. However, due to their complexity, it is challenging to relate a specific symbiotic phylotype to its corresponding function. In the present study, we focused on the forest cockchafer (*Melolontha hippocastani*), a phytophagous insect with a dual life cycle, consisting of a root-feeding larval stage and a leaf-feeding adult stage. By combining *in vivo* stable isotope probing (SIP) with ^{13}C cellulose and ^{15}N urea as trophic links, with Illumina MiSeq (Illumina-SIP), we unraveled bacterial networks processing recalcitrant dietary components and recycling nitrogenous waste. The bacterial communities behind these processes change between larval and adult stages. In ^{13}C cellulose-fed insects, the bacterial families Lachnospiraceae and Enterobacteriaceae were isotopically labeled in larvae and adults, respectively. In ^{15}N urea-fed insects, the genera *Burkholderia* and *Parabacteroides* were isotopically labeled in larvae and adults, respectively. Additionally, the PICRUSt-predicted metagenome suggested a possible ability to degrade hemicellulose and to produce amino acids of, respectively, ^{13}C cellulose- and ^{15}N urea labeled bacteria. The incorporation of ^{15}N from ingested urea back into the insect body was confirmed, in larvae and adults, by isotope ratio mass spectrometry (IRMS). Besides highlighting key bacterial symbionts of the gut of *M. hippocastani*, this study provides example on how Illumina-SIP with multiple trophic links can be used to target microorganisms embracing different roles within an environment.

Keywords: ***Melolontha hippocastani*, nitrogen recycling, cellulose degradation, gut bacteria, symbiotic bacteria, Illumina-SIP, IRMS**

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INTRODUCTION

Symbiotic associations between insects and gut-dwelling microorganisms, especially bacteria, have long been known (Baumberger, 1919), but it was not until the development of next-generation sequencing techniques that we realized the true extent of the diversity and complexity of such microbial communities (Shi et al., 2010). Only one major phylotype is present in insects of the Alydae family, whereas termites can harbor hundreds of major bacterial taxa (Engel and Moran, 2013). A relationship between community composition and host insect taxon and diet has been demonstrated (Colman et al., 2012; Jones et al., 2013; Yun et al., 2014; Guerrero et al., 2016), suggesting crucial roles of the symbionts with regard to insect physiology (Potrikus and Breznak, 1981; Douglas, 2009; Watanabe and Tokuda, 2010).

Cleveland demonstrated for the first time that termite gut symbionts are essential for the digestion of cellulose and for the viability of the host (Cleveland, 1924). Since then, the contribution of insect symbionts in cellulose digestion (Rössler, 1961; Bayon and Mathelin, 1980; Martin, 1983; Anand et al., 2010) and, more recently, in the recycling of nitrogen (Potrikus and Breznak, 1981; Sasaki et al., 1996; Sabree et al., 2009; Thong-On et al., 2012; Ayayee et al., 2014) have been object of extensive research. In insects possessing specialized hindguts chambers such as termites or scarabaeid beetles, cellulose breakdown occurs thanks to the combined action of host- and microbe-secreted enzymes (Watanabe and Tokuda, 2010; Calderon-Cortes et al., 2012). To which extent the host contributes to the recycling of nitrogen is still unclear; however, a shared pathway has been reported in the cockroach *Blatella germanica* (Patiño-Navarrete et al., 2014).

Nonetheless, the insect digestive tract is a complex ecosystem that renders challenging to relate specific microorganisms to their role within the community. Studies involving bacterial isolation allowed the identification of some cellulolytic and uricolytic bacteria (Bridges, 1981; Anand et al., 2010; Huang et al., 2012; Morales-jiménez et al., 2013), but the lack of culturability of the majority of gut microorganisms and the impossibility of accurately reproducing in artificial media the natural conditions of the digestive tract make cumbersome to assess the real significance of such *in vitro* observations in an *in vivo* scenario. The combination of stable isotope probing (SIP) with culture-independent methods of microorganism identification has proven to be a reliable and straightforward approach that allows to link a particular function to specific community members (Lu and Conrad, 2005; Bell et al., 2011; Reichardt et al., 2011; Wüst et al., 2011). By introducing the isotopically labeled substrate of interest into the otherwise unmodified environment, it is possible to disclose an organism's ability to incorporate the heavy isotope into its nucleic acids, thus directly relating certain bacterial taxa to the use of a specific compound (Dumont and Murrell, 2005). In order to increase sensitivity, SIP can be coupled with high throughput amplicon sequencing methods such as Pyrosequencing or Illumina (Aoyagi et al., 2015). This combination has been applied to determine the relevant microorganisms in diverse environments (Paes et al., 2015; Liu et al., 2017) and within the gut of mammals (Berry et al., 2013; Godwin et al., 2014) or insects (Shao et al., 2014).

Our object of study is the gut symbiotic bacteria of the forest cockchafer (*Melolonthahippocastani*), a beetle belonging to the Scarabaeidae family. During its entire life cycle, this insect feeds on living plants, either on the roots during the underground larval stage or on the leaves in the aboveground adult stage, causing significant damage to vegetation during occasional population outbreaks (Jackson and Klein, 2006). The composition of the intricate bacterial community that populates the digestive tract of *M. hippocastani* remained unknown until the works of Arias-Cordero et al. (2012) and Alonso-Pernas et al. (2017), as previous studies in scarabaeid microbiome focused on other beetles (Egert et al., 2003; Zhang and Jackson, 2008; Franzini et al., 2016) or its closely related species, *Melolontha melolontha* (Egert et al., 2005). These works have shown that, at the class level, the taxonomic composition of the gut community of *M. hippocastani* remains constant across the entire insect life cycle, notwithstanding the variation in habitat and nourishment between larval and adult stages (Arias-Cordero et al., 2012; Alonso-Pernas et al., 2017).

Since these purely descriptive studies did not allow inference about the function of the gut symbionts, in the present study we combined DNA isotopic labeling with Illumina MiSeq in order to shed some light on the actual role of the gut bacteria in key nutritional processes of phytophagous insects, namely the degradation of cellulose and the recycling of nitrogen. For those purposes, we fed the insects with diet mixed with either ^{13}C cellulose, or ^{15}N urea, a component of insect nitrogenous waste (Bursell, 1967; Ayayee et al., 2014). Considering the constancy of *M. hippocastani* symbiotic community, we expected to unmask a taxonomic shift in the active microbial players coupled with the transition from larval to adult stage, as a result of adaptation to the radically new diet. By isotope ratio mass spectrometry (IRMS) analyses of host tissues, we aimed to detect the incorporation of ^{15}N from urea into the insect body, thus confirming the existence of a nitrogen-recycling mechanism (Potrikus and Breznak, 1981). Additionally, by introducing the obtained sequencing data of the 16S rRNA genes to the PICRUSt software, which predicts the functional composition of a metagenome using marker gene data and reference genomes (Langille et al., 2013), we produced predictions of the mechanisms that might be behind the above-mentioned processes.

MATERIALS AND METHODS

Insect Collection, Feeding, and DNA Extraction

Second-instar (L2) larvae were collected in a deciduous forest next to Pfungstadt (Germany, 49°49′44″ N 8°36′17″ E) in June 2016. Adult insects were collected in a deciduous forest next to Hanau (Germany, 50°07′02″ N 8°59′26″ E) in May 2016. Insects were transported to the laboratory in boxes with soil or three leaves. ^{12}C cellulose, ^{14}N urea, and ^{15}N urea were purchased from Sigma-Aldrich (St. Louis, MO, USA). ^{13}C cellulose purified from potato was purchased from IsoLife (Wageningen, The

Netherlands). Upon arrival, larvae were transferred to autoclaved sterile plant soil and fed with grated carrot mixed with the experimental compound at a final concentration in the diet of 4 mM (urea) (Ayayee et al., 2014) and 60 mM (cellulose). Optimal cellulose concentration in the diet was estimated based on data from previous labeling experiments with ^{13}C glucose (Alonso-Pernas, unpublished data). Adults were fed with ethanol-sterilized oak leaves overlaid with an aqueous solution of urea or a cellulose suspension at the same final concentrations mentioned above. For each substrate and life stage, a treatment group was fed with isotopically labeled compound, and a control group was fed with unlabeled compound. A group was composed of three insects. Each insect was fed separately in an individual compartment within a climate chamber simulating natural conditions of light, temperature and humidity, for 7 days (urea) and 5 days (cellulose). By using long feeding time we aimed to unveil not only the primary degraders of the substrate, but also the subsequent secondary utilizers that process the derived metabolites (Neufeld et al., 2007; Shao et al., 2014). After feeding, insects were frozen and stored at −80°C until further processing. Before dissection, insects were thawed on ice and surface-sterilized by being soaked briefly in 70% ethanol and sterile distilled water. Dissection was carried out in phosphate-buffered saline (PBS) solution, on ice. Composition of PBS solution used (per liter) is as follows: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 (pH 7.4). Whole guts were carefully extracted and dried in a Concentrator 5301 Speedvac (Eppendorf, Hamburg, Germany) at 45°C for 90 min, and then crushed with a sterile pestle. 5 mg of dry powdered gut tissue was used for DNA extraction with the MasterPure™ Complete DNA and RNA Purification Kit (Illumina, San Diego, CA, USA) according to manufacturer's instructions. The concentration and purity of extracted DNA were checked with NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA was stored at −20°C until further processing.

DNA Separation in CsCl Density Gradient and Fractionation

CsCl gradients were prepared as described (Neufeld et al., 2007). In short, ∼2 μg of purified DNA was mixed with 4.8 ml of 7.163 M CsCl aqueous solution and the corresponding volume of gradient buffer (0.1 M Tris, 0.1 M KCl, and 1 mM EDTA) to achieve a final density of 1.725 g/ml. Solution was transferred to 5.1 ml polyallomer tubes (Beckham Coulter, Brea, CA, USA) and centrifuged in an Optima™ L-90K ultracentrifuge fitted with a NVT 90 rotor (Beckham Coulter, Brea, CA, USA) for 40 h at 173,000 g for cellulose gradients, and 66 h at 150,000 g for urea gradients, as longer centrifugation times at lower speeds enhance DNA separation in 15N-labeled gradients (Cadisch et al., 2005). All samples from the same substrate were set up in the same CsCl batch and run in parallel to minimize potential variations. After centrifugation, 400 μL (cellulose) and 200 μL (urea) fractions were collected drop-wise from the bottom of the tube (total of 12 fractions for cellulose gradients, 24 for urea gradients). The density of each fraction was determined by weighing per triplicate a volume of 100 μL in a fine-scale balance. DNA was precipitated with polyethylene glycol (PEG) 6000 (VWR International, Radnor, PA, USA), washed twice with 70% ethanol and resuspended in sterile double-distilled water.

DNA Concentration Measurement and qPCR of Selected Density Ranges

The DNA-density profile for each gradient was assessed using the Helixyte Green™ Nucleic Acid Stain (Bioquest, Bengaluru, India), according to the protocol provided by the manufacturer. The fluorescence was measured with an Infinite F200 PRO plate reader (Tecan, Männedorf, Switzerland), and DNA concentration for each fraction was inferred based on a standard curve. For each experimental compound, equal density ranges showing visible difference in amount of DNA between treatments were selected, and fractions within each range were grouped together in pooled fractions (upper, medium (upper medium and lower medium in urea gradients) and lower) (**Figure 1**). The 16S rRNA gene copy number for each pooled fraction was determined by quantitative real-time PCR (qPCR) (Lueders et al., 2004) using the 16S rRNA gene specific primers Bact 519F (5′-CAG CMG CCG CGG TAA NWC-3′) and Bact 907R (5′-CCG TCA ATT CMT TTR AGT T-3′) (Stubner, 2002). Reactions contained 2 μL of template DNA, primers at a concentration of 0.3 mM each and 1x Brilliant III SYBR® Green QPCR Master Mix (Agilent, Santa Clara, CA, USA), making a total volume of 20 μL. The program consisted of a 95°C hold for 5 min followed by 40 cycles of 45 s at 95°C, 30 s at 50°C and 50 s at 72°C. PCRs were carried out in a CFX96™ Real-Time PCR Detection System (BioRad, Hercules, CA, USA). *E. coli* genomic DNA was used to relate quantitative cycle values to the 16S rRNA gene copy number of each reaction (Supplementary Figure 1). In order to correct for differences in amount of recovered DNA and allow comparison between control and labeled gradients, fraction DNA and 16S rRNA gene copy number were normalized as percentage of, respectively, the total amount of DNA or 16S rRNA copy number of all the fractions within a gradient.

Illumina Sequencing, Data Analysis, and Metagenome Reconstruction

Pooled fractions were submitted for 16S rRNA gene Illumina MiSeq to Molecular Research Laboratory (www.mrdnalab.com; Shallowater, TX, USA). In short, the 16S rRNA gene V4 variable region was amplified using the PCR primers 28F (5′-GAG TTT GAT CNT GGC TCA G-3′) and 519R (5′-GTN TTA CNG CGG CKG CTG-3′). After amplification, PCR products were checked in 2% agarose gel and purified using Agencourt AMPure beads (Agencourt Bioscience Corporation, Beverly, MA, USA). The purified PCR products were used to prepare the DNA library following the Illumina MiSeq DNA library preparation protocol using the MiSeq reagent kit V3 (2X300 bp) for paired-end reads following the manufacturer's guidelines. The quality control and analysis of Illumina reads was done in QIIME version

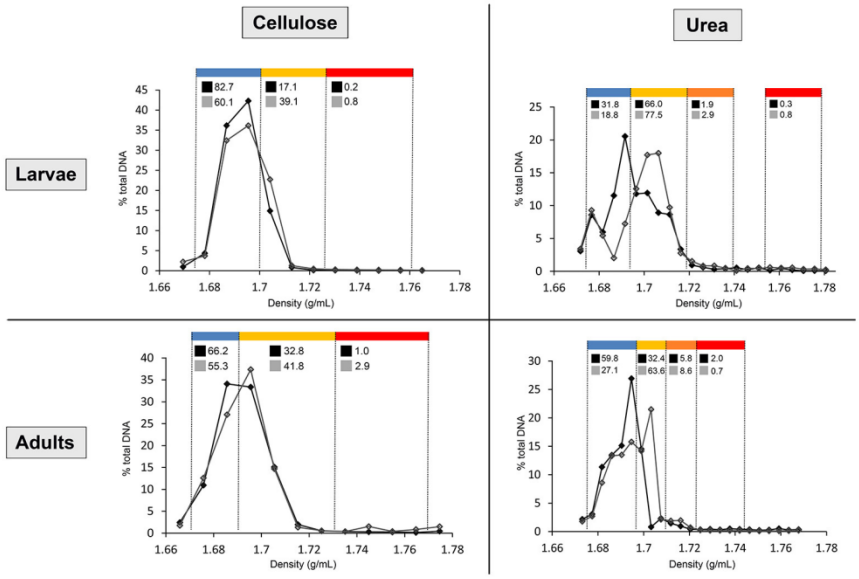

FIGURE 1 | Distribution of the DNA in cellulose and urea gradients, in larvae and adults. Line chart shows the percentages of total gradient DNA contained in each fraction of control gradient (black line, full markers) and isotopically labeled gradient (gray line, empty markers). Density ranges constituting each pooled fraction are indicated in blue (upper fraction), yellow (medium fraction in cellulose gradients, upper medium fraction in urea gradients), orange (lower medium fraction in urea gradients), and red (lower fraction). Numbers above the line chart show the percentages of total gradient 16S rRNA gene copy number contained in each pooled fraction of control gradient (black square) and labeled gradient (gray square).

1.8.0 (Caporaso et al., 2010a). Low-quality reads (quality cut-off = 25) and sequences <200 bp in length were removed, and the remaining reads were denoised using the "denoiser" algorithm as implemented in QIIME (Reeder and Knight, 2010). Denoised high-quality reads were clustered into operational taxonomic units (OTUs) using the *pick_open_reference_otus.py* script and uclust (Edgar, 2010) with 97% nucleotide identity cut-offs. For each OTU, the most abundant sequence was chosen as a representative sequence and aligned to the newest Greengenes core set available (http://greengenes.lbl.gov/) (McDonald et al., 2012) using PyNast (Caporaso et al., 2010b). RDP classifier was used for taxonomy assignment (Wang et al., 2007). An OTU table was constructed describing the abundance of each bacterial phylotype in each pooled fraction. Abundances were normalized as percentages of total sequences in order to correct for differences in library sizes. Based on previous research, activity of a bacterial taxon was defined as its increase in percentage of relative abundance (enrichment) in a dense pooled fraction (medium or lower) of the labeled gradient compared to the same pooled fraction in the control (unlabeled) gradient (Lu and Conrad, 2005; Wüst et al., 2011; Shao et al., 2014):

$$A = \left[\frac{tNS_{labeled\ pooled\ fraction}}{NS_{labeled\ pooled\ fraction}} \cdot 100\right] - \left[\frac{tNS_{control\ pooled\ fraction}}{NS_{control\ pooled\ fraction}} \cdot 100\right]$$

A stands for activity, tNS stands for number of sequences belonging to a certain taxon, NS stands for total number of sequences. One activity value was obtained for each dense pooled fraction (two for cellulose gradients, three for urea gradients). Identification of taxa that were significantly enriched in dense, labeled pooled fractions (active taxa) was carried out using a Fisher Exact Test with FDR correction in METASTATS (White et al., 2009). The significance threshold was set at $p = 0.05$, but taxa active with $p < 0.1$ were also considered for discussion as tending to be significant. Heatmaps representing level of activity were constructed using the MultiExperiment Viewer (MeV) software (Saeed et al., 2003). Phylogenetic trees were calculated using the maximum likelihood method (General Time Reversible model) with 1,000 bootstrap replicates in MEGA6 (Tamura et al., 2013). The bacterial metagenome of medium and lower pooled fractions was reconstructed using the PICRUSt software (Langille et al., 2013), using as the input file a PICRUSt-compatible closed-reference OTU table generated from the above-mentioned open-reference OTU table with the *filter_otus_from_otu_table.py* script. Non-labeled families were filtered out of the OTU table using the *filter_taxa_from_otu_table.py* script. 16S rRNA copy numbers per OTU were normalized with the *normalize_by_copy_number.py* script and IMG database information. The metagenome inference was performed with the *predict_metagenomes.py* script, using the normalized OTU table as input. NSTI values, a measure of prediction uncertainty, were also calculated (Supplementary Table 1). Certain KEGG Orthologs were selected according to their relationship with the experimental substrate (lignocellulose digestion for cellulose,

nitrogenous waste degradation and amino acid production for urea). Calculations of the enrichment of KEGG Orthologs in dense isotopically-labeled pooled fractions, reflecting their presence in labeled bacterial families, and construction of heatmaps were done as described above for activity of bacterial taxa.

Determination of the Level of ^{15}N Isotopic Enrichment of Insect Tissues

To determine the level of ^{15}N enrichment in the insect tissue, larvae, and adults were dissected as described above to excise the tissues of interest (gut, fat bodies, and muscular tissue). Tissues were rinsed in sterile PBS before being dried and crushed. For each life stage, three control insects fed with ^{14}N urea and three treated insects fed with ^{15}N urea were used for the measurement. Three technical replicates of each biological replicate were analyzed. The abundance of the ^{15}N was determined by a coupled elemental analyzer/isotope ratio mass spectrometry (EA/IRMS) and is given in δ-notation (*vide infra*). About 0.3 mg of dry, powdered tissue was weighed with an ultra-micro balance (UMX2, Mettler-Toledo) in small 0.04 ml tin capsules (3.5 × 5 mm, HEKATech, HE 24005300). The capsules were sealed and combusted (oxidation at 1,020°C, reduction at 650°C) in a constant helium stream (80 ml/min) quantitatively to CO_2, N_2, and H_2O using an elemental analyzer (EuroEA CN2 dual, HEKAtech GmbH, Wegberg, Germany). After passing through a water trap ($MgClO_4$) the gases were separated chromatographically at 85°C and transferred via an open split to a coupled isotope ratio mass spectrometer (IsoPrime, Micromass, Manchester, UK). Isotope ratios were generally calculated as:

$$\delta^N E = \left[\frac{(R_{sample} - R_{standard})}{R_{standard}}\right] - 1$$

δ values usually are small numbers. Hence, they are commonly multiplied by 1,000 and communicated in ‰units or mUr (Brand and Coplen, 2012). N is the heavy isotope of the element E, R is the ratio of heavy to light isotope ($^{15}N/^{14}N$) of the sample and the standard, respectively. δNE is the relative deviation of the heavy to light isotope ratio from the international standard (air-N_2 for nitrogen). Samples were measured against our laboratory working standard alice-1 (acetanilide, $\delta^{15}N = -1.44 \pm 0.12$‰), which has been calibrated for δ15N by a two-point normalization using IAEA-N1 (+0.43‰), and IAEA-N2 (+20.40‰) (Böhlke and Coplen, 1995). Empty tin capsules were used as blanks. A caffeine standard (cafice-1, $\delta^{15}N = -4.01 \pm 0.10$‰) was analyzed together with the samples as quality assurance reference material for long-term performance monitoring of the whole analytical setup (Werner and Brand, 2001). $\delta^{15}N$-values of enriched samples are not corrected for m/z = 30 trace ($^{15}N_2$). Since number of biological replicates ($n = 3$) was too small to test for normal distribution, the statistical significance of differences in δ values was calculated using the Mann–Whitney U test (significance threshold $p = 0.05$) in Prism 4 software (GraphPad Software Inc., La Jolla, CA, USA). Data are given as mean +/− SEM (Standard Error of Mean).

Nucleotide Sequence Accession Numbers

The Illumina raw sequencing data were deposited at the NCBI GenBank Short Read Archive under accession numbers SRR5296204 (^{12}C and ^{13}C cellulose gradients) and SRR5296203 (^{14}N and ^{15}N urea gradients).

RESULTS

Isopycnic Centrifugation and Separation of Light and Heavy DNA

After centrifugation, between 500 and 1,000 ng of DNA were recovered from the gradients by PEG precipitation. In order to normalize the variation in amount of recovered DNA between gradients, fraction's DNA concentration and 16S rRNA gene copy number are expressed as percentages of total amount within the gradient. For urea, shifting of the DNA toward dense parts (>1.69 g/mL) of the labeled gradient (^{15}N) compared to control (^{14}N) is already observable in the quantification carried out with Helyxyte green. qPCR performed with bacteria-specific primers clarified the increase of 16S rRNA gene copy number in dense pooled fractions (medium and lower) of the isotopically labeled gradient (^{13}C or ^{15}N) in comparison to the unlabeled control gradient (^{12}C or ^{14}N), confirming successful labeling for both substrates (**Figure 1**).

Illumina MiSeq Analysis of Bacterial Diversity in Pooled Fractions and Assessment of Relative Activity

After processing raw Illumina MiSeq data, 145,737 (cellulose larvae) 134,580 (cellulose adults) 367,589 (urea larvae) 531,465 (urea adults) high quality reads were obtained. The sequence analysis showed an increase of the relative abundance of particular bacterial taxa in the dense pooled fractions (medium and lower) of the isotopically labeled gradient (^{13}C or ^{15}N) compared to those in the control gradient (^{12}C or ^{14}N). This indicates the incorporation of the heavy isotope into the bacterial DNA (labeling). Based on the variation in relative abundance between dense pooled fractions of the labeled gradient and those of the control gradient, the activity value of each bacterial taxon for each experimental substrate was determined.

In larvae fed on diet supplemented with cellulose, the detected families showing activity were Lachnospiraceae and Enterococcaceae ($p < 0.1$), Desulfovibrionaceae, Ruminococcaceae, Bacteroidaceae, unclassified families in the order Clostridiales, unclassified families in the class Alphaproteobacteria, Christensenellaceae, and Acetobacteraceae (non-significant) (**Figure 2**). However, none of them achieved statistically significant labeling ($p < 0.05$), possibly due to occurence of a strong interspecific competition for the experimental substrate, slow growth and/or usage of other carbon sources besides cellulose. In adults, detected active families were Enterobacteriaceae ($p < 0.01$), and Enterococcaceae, Desulfovibrionaceae, Ruminococcaceae, and Porphyromonadaceae (non-significant) (**Figure 3**). The highly significant activity of the Enterobacteriaceae family suggests that, in contrast to larvae, a single family might undertake

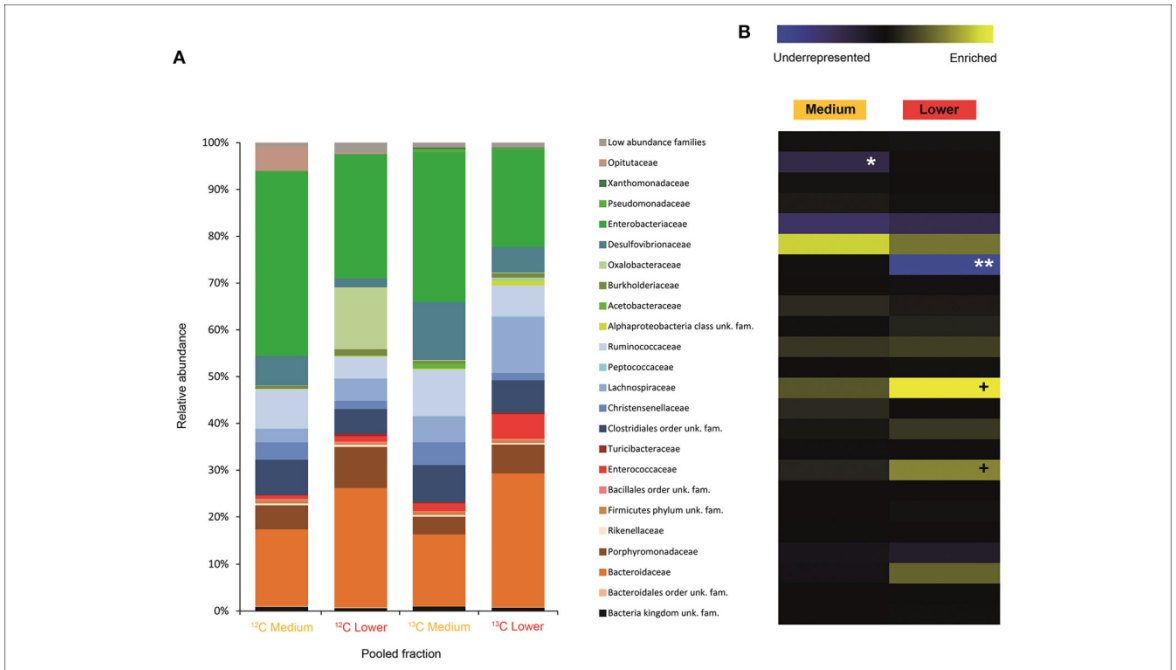

FIGURE 2 | Result of the Illumina MiSeq sequencing of the gradient constructed with DNA from larvae fed on diet supplemented with cellulose (145, 737 sequences in total). The relative abundance of bacterial families in dense pooled fractions of ^{12}C and ^{13}C gradients, in percentage of total sequences, is displayed in **(A)**. The enrichment or underrepresentation of bacterial families in the medium and lower pooled fractions of the ^{13}C gradient compared to the same fractions in the ^{12}C gradient, as a measure of the activity in the processing of cellulose, is shown in **(B)**. Blue represents underrepresentation (inactive families), while yellow represents enrichment (active families). $^{**}p < 0.01$; $^{*}p < 0.05$; $+ p < 0.1$.

the degradation of cellulose in the adult gut. Interestingly, no appreciable labeling of the Enterobacteriaceae family was observed in larvae. Lachnospiraceae, the family showing the highest activity value in larvae, is significantly underrepresented in the lower pooled fraction of labeled adult gradient ($p < 0.01$). This suggests the occurrence of an expected taxonomic shift in the active bacteria across the host's developmental stages. Not all the OTUs could be classified at the genus level. Nevertheless, among the identified genera, the genus *Trabulsiella* (Enterobacteriaceae family) showed the highest activity in larvae ($p < 0.05$). In adults, the genus *Enterococcus* (Enterococcaceae family) had the highest activity, although not statistically significant (**Table 1**). Maximum likelihood trees of the most active of the identified genera with closely related NCBI retrieved sequences are shown in Supplementary Figure 2.

Labeling of certain bacterial families was also accomplished using urea as a trophic link, indicating the ability of gut bacterial symbionts to incorporate nitrogen from the insect's waste compounds into nucleic acids. A high number of bacterial families in the larvae were enriched in the medium and lower pooled fractions of the ^{15}N gradient; following are the ones with higher activities values: Burkholderiaceae ($p < 0.01$), Christensenellaceae, Ruminococcaceae and unclassified families in the order Bacillales ($p < 0.1$), unclassified families in the order Clostridiales, Moraxellaceae, Propionibacteriaceae, Xanthomonadaceae, Staphylococcaceae, and unclassified families in the class Bacilli (non-significant) (**Figure 4**). The detected active bacterial families in the adults were Porphyromonadaceae and Bacteroidaceae ($p < 0.05$), Ruminococcaceae ($p < 0.1$), Desulfovibrionaceae, and Enterobacteriaceae (non-significant) (**Figure 5**). Again, bacteria were differently labeled when comparing larvae and adults, suggesting that the taxonomic shift in active communities also happens in symbionts involved in urea processing. Among the OTUs that could be classified to the genus level, *Burkholderia* sp. (99% of the Burkholderiaceae family sequences) showed highly significant activity in larvae ($p < 0.01$). *Parabacteroides* sp. (99% of the Porphyromonadaceae family sequences) and *Bacteroides* sp. (100% of the Bacteroidaceae family sequences) also achieved statistically significant activities in adults ($p < 0.05$) (**Table 2**). Maximum likelihood trees of the most active of the identified genera with closely related NCBI retrieved sequences are shown in Supplementary Figure 2.

PICRUSt Predicted KEGG Ortholog Enrichment in Dense Isotopically Labeled Fractions

After running PICRUSt, the predicted metagenome of each dense pooled fraction (medium and lower) from the isotopically labeled

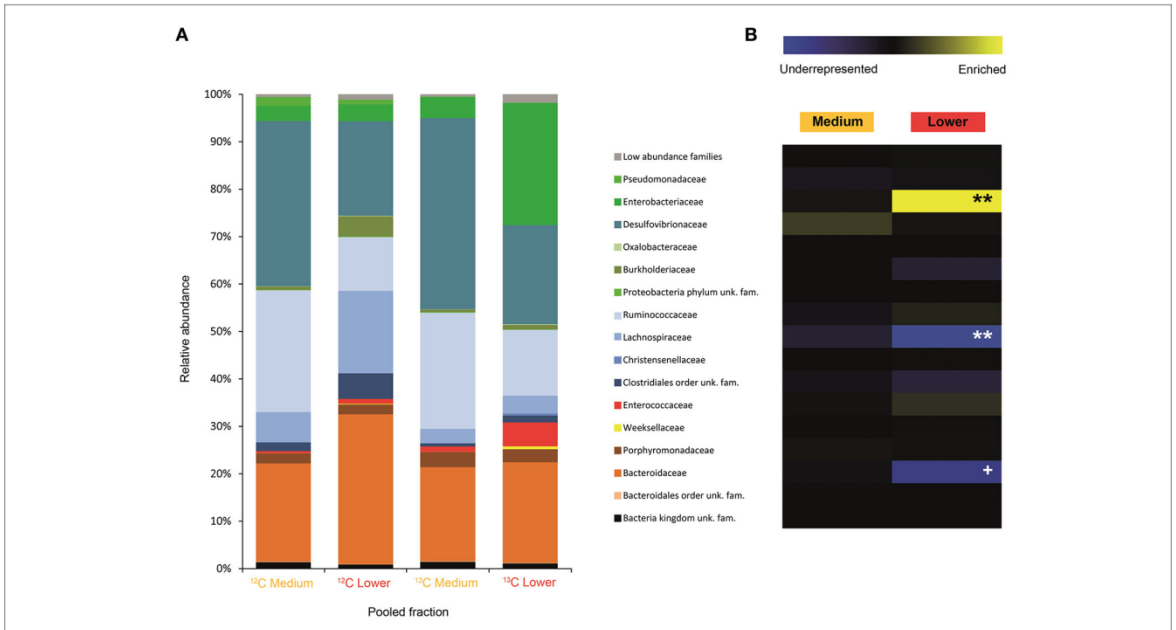

FIGURE 3 | Result of the Illumina MiSeq sequencing of the gradient constructed with DNA from adults fed on diet supplemented with cellulose (134, 580 sequences in total). The relative abundance of bacterial families in dense pooled fractions of ^{12}C and ^{13}C gradients, in percentage of total sequences, is displayed in **(A)**. The enrichment or underrepresentation of bacterial families in the medium and lower pooled fractions of the ^{13}C gradient compared to the same fractions in the ^{12}C gradient, as a measure of the activity in the processing of cellulose, is shown in **(B)**. Blue represents underrepresentation (inactive families), while yellow represents enrichment (active families). $^{**}p < 0.01$; $^{+}p < 0.1$.

TABLE 1 | Bacterial genera showing the highest activity values among the identified genera in larvae and adults fed on diet supplemented with cellulose.

Cellulose							
Larvae				Adults			
Family	**Genus**	**Med**	**Low**	**Family**	**Genus**	**Med**	**Low**
Enterobacteriaceae	*Trabulsiella*	7.0*	4.0	Enterococcaceae	*Enterococcus*	−0.1	2.8
Porphyromonadaceae	*Parabacteroides*	1.9	3.1	Porphyromonadaceae	*Parabacteroides*	1.0	0.7
Bacteroidaceae	*Bacteroides*	−1.0	3.0	Enterobacteriaceae	*Serratia*	0.0	1.4
Acetobacteraceae	*Gluconobacter*	1.1	0.6	Enterobacteriaceae	*Erwinia*	0.0	1.2
Enterobacteriaceae	*Serratia*	0.8	0.4				

*Activity values of each genus in medium and lower pooled fractions is showed in Med and Low columns, respectively. *$p < 0.05$.*

gradient (^{13}C or ^{15}N) was compared to that in the control gradient (^{12}C or ^{14}N). Heatmaps were constructed showing the enrichment or underrepresentation of KEGG Orthologs dense pooled fractions of the isotopically labeled gradients, as an indication of its presence or absence among isotopically labeled bacterial families.

We considered 39 KEGG Orthologs involved in lignocellulose degradation for insects fed with a diet supplemented with cellulose (Supplementary Figure 3). Only the most significantly active families detected by Illumina-SIP were considered in the metagenomic prediction (Lachnospiraceae and Enterococcaceae in larvae, Enterobacteriaceae in adults). KEGG Orthologs involved in cellulose and hemicellulose degradation were the most enriched in labeled lower pooled fraction in both larvae and adults, suggesting that the cellulose-labeled families might be able to process hemicellulose as well. Some KEGG Orthologs potentially involved in lignin digestion were present only in the labeled bacterial family from adults (Enterobacteriaceae).

For insects fed with a diet supplemented with urea, we considered 49 KEGG Orthologs involved in nitrogenous waste degradation (uric acid and urea) and in the production of amino acids from ammonia (NH_3), the end product of uric acid and urea degradation, along with CO_2 (Supplementary Figure 4). Only the most significantly active bacterial families detected by

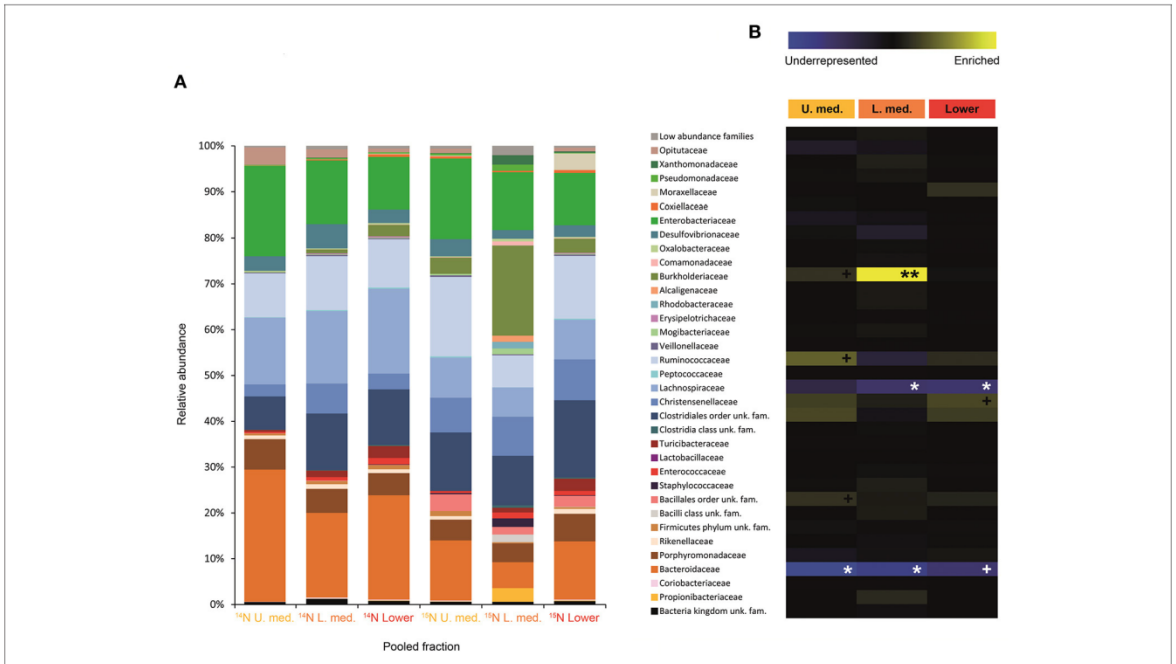

FIGURE 4 | Result of the Illumina MiSeq sequencing of the gradient constructed with DNA from larvae fed on diet supplemented with urea (367, 589 sequences in total). The relative abundance of bacterial families in dense pooled fractions of ^{14}N and ^{15}N gradients, in percentage of total sequences, is displayed in **(A)**. The enrichment or underrepresentation of bacterial families in the medium and lower pooled fractions of the ^{15}N gradient compared to the same fractions in the ^{14}N gradient, as a measure of the activity in the processing of urea, is shown in **(B)**. Blue represents underrepresentation (inactive families), while yellow represents enrichment (active families). $^{**}p < 0.01$; $^{*}p < 0.05$; $^{+}p < 0.1$.

Illumina-SIP were considered in the metagenomic prediction (Burkholderiaceae in larvae, Porphyromonadaceae in adults). The pathways leading to the synthesis of amino acids from free ammonia appear to be enriched in urea-labeled bacteria from adults and larvae, supporting their ability to incorporate waste nitrogen back into amino acids. The uricolytic and ureolytic pathways, however, appear to be only barely enriched in labeled bacterial families from larvae. This brings up the possibility of a cross-feeding between ammonia and amino acid producers.

Determining the Level of Isotopic Enrichment of Insect Tissue

Isotope Ratio Mass Spectrometry (IRMS) measurements of selected insect tissues (gut, fat bodies and muscular tissue) showed the statistically significant incorporation of ^{15}N into the insect bodies of both larvae and adults (**Figure 6**), suggesting host ability to reuse waste nitrogen in both life stages.

DISCUSSION

Symbiotic Community Involved in the Processing of Cellulose in Larvae and Adults

Symbiotic bacterial families involved in the processing of cellulose were detected by combining data from SIP and Illumina MiSeq. The less evident DNA density shift in cellulose gradients compared to urea is probably due to the lower speed and longer centrifugation time used (Cadisch et al., 2005), coupled with the higher resolution achieved by splitting urea gradients in 24 fractions instead of 12. As hypothesized in the introduction, Illumina sequencing results showed that the taxonomic affiliation of the active community depended on the stage of insect development, and it involved not only dominant bacterial groups but also minor ones. Influence of soil environmental bacteria on the observed shift is negligible, as gut community composition of *M. hippocastani* larvae shows no overlap with that of the surrounding soil (Arias-Cordero et al., 2012). By feeding the insects during 5 days, we aimed to capture as much as possible of the active community involved in the trophic network of cellulose degradation, not only the primary consumers. When interpreting the results, the limitations of SIP must be taken into account: non-detectable labeling of a certain bacterial family may indicate that they are not able to use cellulose or any of its degradation products, but also it might be a consequence of insufficient label incorporation due to (a) slow growth or (b) reliance on other carbon sources besides cellulose. In larvae, the families Lachnospiraceae, Enterococcaceae, and Desulfovibrionaceae showed the highest activities in terms of cellulose processing (**Figure 2**). These are anaerobic, fermentative families, although some Desulfovibrionaceae and Enterococcaceae members are

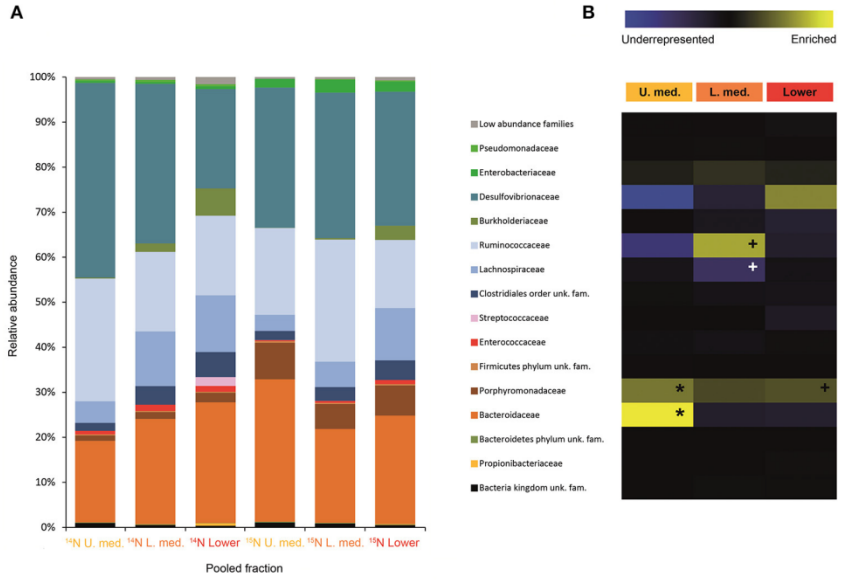

FIGURE 5 | Result of the Illumina MiSeq sequencing of the gradient constructed with DNA from adults fed on diet supplemented with urea (531, 465 sequences in total). The relative abundance of bacterial families in dense pooled fractions of ^{14}N and ^{15}N gradients, in percentage of total sequences, is displayed in **(A)**. The enrichment or underrepresentation of bacterial families in the medium lower pooled fractions of the ^{15}N gradient compared to the same fractions in the ^{14}N gradient, as a measure of the activity in the processing of urea, is shown in **(B)**. Blue represents underrepresentation (inactive families), while yellow represents enrichment (active families). $^{*}p < 0.05$; $^{+}p < 0.1$.

able to cope with oxygen (Cypionka, 2000; Felis et al., 2015). The Lachnospiraceae family display cellulolytic capabilities (Sharma et al., 2015), and its abundance in the gut of the scarabaeid *Holotrichia parallela* is enriched by woody diets (Huang et al., 2013). Moreover, genomic analyses unveiled presence of butyrate production genes in this family (Vital et al., 2014) and evidence points to them as potential acetogens (Gagen et al., 2015). Thus, the Lachnospiraceae family probably contributes to the fatty acid pool of the gut. Cellulases are produced by Enteroccoccaceae members as well (Shil et al., 2014), and it has been demonstrated that presence of *Enterococcus faecalis* in the digestive tract increases the amount of food consumed by a beetle host, presumably due to its cellulolytic activity (Schmid et al., 2014). Short-chain fatty acids resulting of fermentative processes, such as acetate, propionate or butyrate, may be absorbed through the gut epithelium, and used by the insect as energy source via citrate cycle or carbon source for the synthesis of fatty acids (Bayon, 1980; Bayon and Mathelin, 1980; Odelson and Breznak, 1983; Terra and Ferreira, 2009). Desulfovibrionaceae might have incorporated some labeling due to cross-feeding interaction with primary cellulose consumers, as reported for termite guts, where members of this family oxidize a variety of fermentation products to acetate (Dröge et al., 2005). Desulfovibrionaceae might also enhance cellulose degradation by balancing the redox potential of the gut content and contributing to the maintenance of anoxic conditions (Bharati et al., 1982; Dröge et al., 2005; Arias-Cordero et al., 2012). Collectively, these observations suggest that the families Lachnospiraceae, Enterococcaceae, and Desulfovibrionaceae might work in synergy in the larval gut, Lachnospiraceae and Enterococcaceae breaking down root organic substrates and making them suitable for the host's digestion, absorption and metabolism, and Desulfovibrionaceae maintaining optimal conditions for this process. Host-produced cellulases released in the midgut may also participate in cellulose degradation (Watanabe and Tokuda, 2010; Calderon-Cortes et al., 2012), although the short residence time of the food in the midgut (4–8 h in *M. melolontha*) compared to the hindgut (up to 4 days) (Wildbolz, 1954) suggest that the bulk of digestion of recalcitrant compounds is carried out in the hindgut. Although the families Lachnospiraceae and Desulfovibrionaceae are abundant in the larval gut (Egert et al., 2005; Arias-Cordero et al., 2012), Enterococcaceae seems to be minor (Alonso-Pernas et al., 2017). This reflects the role of secondary bacterial phylotypes, which are easily overlooked, in major gut processes such as cellulose digestion. At the genus level, *Trabulsiella* sp. (Enterobacteriaceae family) showed the highest activity of the identified genera (**Table 1**). The type species of this genus, *Trabulsiella guamensis*, shows ability to ferment a wide variety of carbohydrates, including glucose and cellobiose (Janda, 2006), and genomic analyses of five strains of *Trabulsiella* sp. isolated from the wood-feeding termite *Heterotermes* sp. showed the presence of an array of cellulolytic enzymes, suggesting their

TABLE 2 | Bacterial genera showing the highest activity values among the identified genera in larvae and adults fed on diet supplemented with urea.

Urea									
Larvae					Adults				
Family	**Genus**	**Umed**	**Lmed**	**Low**	**Family**	**Genus**	**Umed**	**Lmed**	**Low**
Burkholderiaceae	*Burkholderia*	3.4	18.4**	1.0	Porphyromonadaceae	*Parabacteroides*	6.4*	3.9	4.4 +
Moraxellaceae	*Enhydrobacter*	0.2	0.0	3.6	Bacteroidaceae	*Bacteroides*	13.5*	−2.3	−2.7
Enterobacteriaceae	*Erwinia*	0.9	1.8	0.5	Enterobacteriaceae	*Gluconacetobacter*	1.0	1.3	1.0
Propionibacteriaceae	*Propionibacterium*	0.1	2.9	0.0					
Porphyromonadaceae	*Dysgonomonas*	1.1	0.4	1.4					
Xanthomonadaceae	*Stenotrophomonas*	0.1	2.0	0.3					
Staphylococcaceae	*Staphylococcus*	0.3	1.8	0.0					
Alcaligenaceae	*Achromobacter*	0.0	1.3	0.0					
Pseudomonadaceae	*Pseudomonas*	0.0	1.0	−0.1					
Rhodobacteraceae	*Rubellimicrobium*	0.0	1.0	0.0					

*Activity values of each genus in upper medium, lower medium and lower pooled fractions is showed in Umed, Lmed and Low columns, respectively. **$p < 0.01$; *$p < 0.05$; +$p < 0.1$.*

involvement in cellulose degradation within the digestive tract (Olvera-García et al., 2015).

In adults, a much less diverse community made up of exclusively the Enterobacteriaceae family (**Figure 3**) was significantly active in the processing of cellulose. At the family level, Enterobacteriaceae was not active in larvae. This family, which is commonly associated with herbivore insects, possesses broad polysaccharide-degrading abilities, including cellulose, pectins, xylan and starch (Anand et al., 2010; Adams et al., 2011; Engel et al., 2012), and yield succinate, acetate, H_2 and CO_2, among other fermentation products (Wüst et al., 2011). As stated before, acetate can be taken up through the gut epithelium and used by the host (Bayon and Mathelin, 1980; Terra and Ferreira, 2009). The involvement of Enterobacteriaceae members in digestive processes during adult stage is not surprising given the increase in abundance of this bacterial family in the adult gut, where it becomes one of the dominant taxa (Arias-Cordero et al., 2012; Alonso-Pernas et al., 2017). The PICRUSt predicted metagenome shows that the Enterobacteriaceae family might be behind the digestion of a wide array of recalcitrant compounds in the adult gut (Supplementary Figure 3). This simplification of the cellulolytic bacterial network in adults compared to larvae may be a consequence of the increased palatability of a foliage-based diet (32 mg lignin/g dry weight, in oak) compared to a root-based diet (128 mg lignin/g dry weight, in oak) (Arias-Cordero et al., 2012). Among the identified genera, *Enterococcus* sp. (Enterococcaceae family) showed the highest activity, yet it was not statistically significant, suggesting that the most active genus in cellulose degradation within the adult gut may remain unidentified (**Table 1**). As discussed previously for larvae, cellulolytic *Enterococcus* spp. are important for the nourishment of the host (Schmid et al., 2014; Shil et al., 2014). *Enterococcus mundtii*, which is phylogenetically closely related to our *Enterococcus* sp. (Supplementary Figure 2), has been identified as the most active bacterium in the gut of the leaf-feeding larvae of *Spodoptera littoralis* (Shao et al., 2014).

Symbiotic Community Involved in the Processing of Urea in Larvae and Adults

Urea is a secondary insect nitrogenous waste compound and also a degradation product of the main compound, uric acid (Bursell, 1967; Ayayee et al., 2014). Thus, by using urea as trophic link, we aimed to target not only urea but also some uric acid degraders within the gut symbiotic community. Additionally, because of long exposition to labeled urea (7 days), we expected to extend the isotopic labeling to secondary substrate consumers, namely amino acid producers, for a better understanding of the trophic network behind the recycling of nitrogen. Labeling was successful in both larvae and adults, indicating the ability of gut bacteria to break down urea and incorporate the heavy isotope into their DNA. Also, IRMS analyses detected the significant incorporation of ^{15}N in insect tissues (**Figure 6**). Taken together, these results strongly indicate the existence of a symbiont-driven nitrogen recycling mechanism in the gut of *M. hippocastani*, as it happens in planthoppers (Sasaki et al., 1996), termites (Potrikus and Breznak, 1981; Thong-On et al., 2012), and cockroaches (Sabree et al., 2009; Patiño-Navarrete et al., 2014). Isotopic labeling was more prominent and widespread among bacterial taxa in larvae than in adults, which is in line with the lower content of organic nitrogen content in the larval diet (roots) compared to the adult diet (leaves) (Dickson, 1989). In larvae, the Burkholderiaceae family showed the highest activity. The Ruminococcaceae and Christensenellaceae families and the Bacillales order tended to be significantly active at much lower levels (**Figure 4**). Members of the Burkholderiaceae family are commonly found in association with insects feeding on diets deficient in amino acids, such as wood (Geib et al., 2009) and phloem (Michalik et al., 2016). *Tetraponema* ants harbor Burkholderiales representatives in a pouch-like specialized compartment surrounded by a network of Malphigian tubules, suggesting the involvement of the bacteria in the processing of insect nitrogenous waste (Borm et al., 2008). The role in nitrogen recycling of the other labeled taxa has not yet been studied, to our knowledge. Nevertheless, their low activity may indicate a cross-feeding interaction in which they

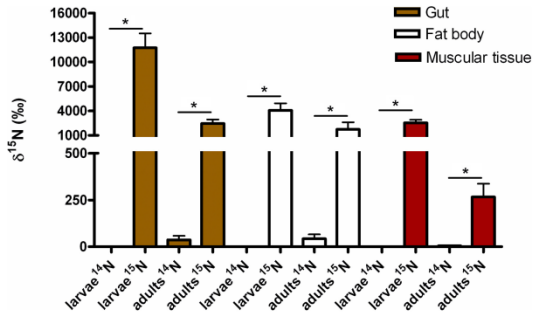

FIGURE 6 | ^{15}N incorporated into body tissues of *M. hippocastani* reared with diet supplemented with ^{15}N urea compared with insects reared with diet supplemented with ^{14}N urea. Results shown are mean values for three replicates per treatment. Error bars represent the standard error of the mean, and asterisks represent statistically significant values with $p = 0.05$.

take up isotopically labeled ammonia excreted by urea-degrading bacteria and use it as nitrogen source for their own growth (Bryant and Robinson, 1961). At the genus level, *Burkholderia* sp. (99% of the Burkholderiaceae family sequences) showed a highly significant activity ($p < 0.01$), pointing it as the most relevant genus within the Burkholderiaceae family (**Table 2**). This genus has been repeatedly found in the digestive tract of insects thriving on nitrogen-deficient diets (Borm et al., 2008; Grünwald et al., 2009; Kikuchi et al., 2010; Reid et al., 2011; Michalik et al., 2016). Some *Burkholderia* strains exhibit nitrogen-fixing ability (Estrada-de los Santos et al., 2001), and the genome of a *Burkholderia*-belonging symbiont encodes complete metabolic pathways for essential amino acids (Shibata et al., 2013). Collectively, these findings suggest *Burkholderia* sp. as a significant bacterium for the recycling of nitrogen in larval *M. hippocastani*.

A change in the active families between larvae and adults indicates that the taxonomic shift is not exclusive to bacteria involved in cellulose digestion, but also happens to bacteria involved in urea processing. The Porphyromonadaceae family showed the highest activity in adults, followed by Bacteroidaceae. Tendency to be significantly active is observed in the family Ruminococcaceae (**Figure 5**). An increase in abundance of the Porphyromonadaceae family and the order Bacteroidales, to which Porphyromonadaceae and Bacteroidaceae belong, has been observed in the hindgut wall of *M. hippocastani* adults compared to L2 larvae. The order Bacteroidales appeared as the most abundant taxon in adult hindgut wall, suggesting a highly significant role (Alonso-Pernas et al., 2017). The Porphyromonadaceae family has been linked to nitrogen fixation in the wood-eating beetle *Odontotaenius disjunctus* (Ceja-Navarro et al., 2014). Another study on the cockroach *Blattella germanica* relates low protein content in the diet with an increase in the abundance of this family in the gut (Perez-Cobas et al., 2015). Moreover, a genomic study of a Bacteroidales bacterium, endosymbiont of termite gut protists, unveiled its potential for synthesizing amino acids from ammonia (Hongoh et al., 2008). Taken together, these results suggest the Porphyromonadaceae family as key bacteria under conditions of nitrogen scarcity, with potential to synthesize amino acids. The genus *Parabacteroides* (99% of the Porphyromonadaceae family sequences) showed the highest activity of the identified genera (**Table 2**). To our knowledge, no report has hitherto addressed the role of this particular genus within the gut, but our data suggest that *Parabacteroides* is the relevant bacterial genus in nitrogenous waste recycling in *M. hippocastani* adults. In second place, the family Bacteroidaceae also showed statistically significant activity. Members of this family might have the genetic potential for the synthesis of amino acids using ammonia from insect waste (Hongoh et al., 2008). In termites belonging to the genus *Reticulitermes*, *Bacteroides* spp. (Bacteroidaceae family) are found among the bacteria responsible for producing ammonia from insect nitrogenous waste (Potrikus and Breznak, 1981). In the present study all the Bacteroidaceae sequences belong to *Bacteroides* sp. Based on these findings, we point the genus *Parabacteroides* as an important waste nitrogen utilizer within the adult *M. hippocastani* gut, possibly with the aid of *Bacteroides* sp. The putative ability of the family Ruminococcaceae to recycle a host's nitrogenous waste remains, to date, unknown. Their low activity makes plausible that they take some of the free ammonia released by primary urea degraders for their own benefit (Bryant and Robinson, 1961).

Surprisingly, none of the bacterial taxa displaying highest activity values in this study have been identified so far as primary degraders of uric acid or urea. In termites, the families Clostridiaceae, Enterobacteriaceae, Streptococcaceae, and Bacteroidaceae are responsible for uric acid degradation (Potrikus and Breznak, 1981; Thong-On et al., 2012). The order Clostridiales and the family Enterobacteriaceae showed non-significant activities in larvae and adults, respectively (**Figures 4**, **5**). The family Bacteroidaceae achieved significant activity in adults (**Figure 5**). In line with these results, the PICRUSt outcome shows that KEGG Orthologs involved in uric acid and urea degradation are barely enriched in the medium and lower pooled fractions of the ^{15}N gradient in both larvae and adults compared to the ^{14}N gradient (Supplementary Figure 4). However, the glutamine synthetase pathway, responsible for the incorporation of free ammonia into glutamate to form glutamine, shows enrichment in both life stages. This might suggest a shared pathway in which urea and uric acid degraders would excrete most of the produced ammonia without incorporating much of the labeled nitrogen, and the bacterial genera discussed above (*Burkholderia* in larvae; *Parabacteroides* and possibly *Bacteroides* in adults) would uptake it for amino- and nucleic acid synthesis. Similar cross-feeding has been reported in a study on the cockroach endosymbiont *Blattabacterium cuenoti*, which degrades urea into ammonia but lacks the ability to use this ammonia through the glutamine synthetase pathway, which is carried out by the host (Patiño-Navarrete et al., 2014). It must be taken into account, however, that such *in silico* predictions are limited by the fact that PICRUSt relies on reference genomes, and does not consider processes such as horizontal gene transfer that may reshape the gene pool of symbiotic bacteria (Hansen and Moran, 2014).

CONCLUDING REMARKS

The microbial community inhabiting the digestive tract of *M. hippocastani* is very complex and consequently problematic to study. We showed how SIP combined with Illumina MiSeq (Illumina-SIP) can be used to underscore the bacterial symbionts involved in relevant processes for the host, thus narrowing down the number of bacterial taxa in which future research should focus on. Our experiments were limited to cellulose processing and the recycling of nitrogen, but it is possible to address other functions of the gut microbiome by using different labeled substrates, for instance, the active bacteria involved in host detoxification processes could be assessed with labeled plant defense compounds. Besides highlighting key microbial symbionts committed to the treatment of cellulose and urea, our data unveiled a shift in their taxonomic affiliation depending on host developmental stage, regardless of the observed community stability throughout the entire insect life cycle. Moreover, low-abundance bacterial phylotypes may be of crucial importance for the gut ecosystem, and PICRUSt predictions suggested possible additional roles for the labeled bacteria, such as production of amino acids and digestion of hemicellulose.

This study set up a starting point for research on the function and dymanics of the gut microbial community of *M. hippocastani*, at the same time it opens interesting questions, such as the elucidation of the molecular mechanisms underlying the bacterial taxonomic shift between host larval and adult stages, the confirmation of the *in silico* PICRUSt results with metagenomic data and to deepen into the individual roles of the active bacterial phylotypes.

ETHICS STATEMENT

Work with insects does not require approval by an ethics committee.

AUTHOR CONTRIBUTIONS

PA, EA, AN, and YS designed the experiments. PA and SB performed the experiments. PA, AN, and LH analyzed the data. PA and AN wrote the manuscript. WB conceived and supervised the project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.01970/full#supplementary-material

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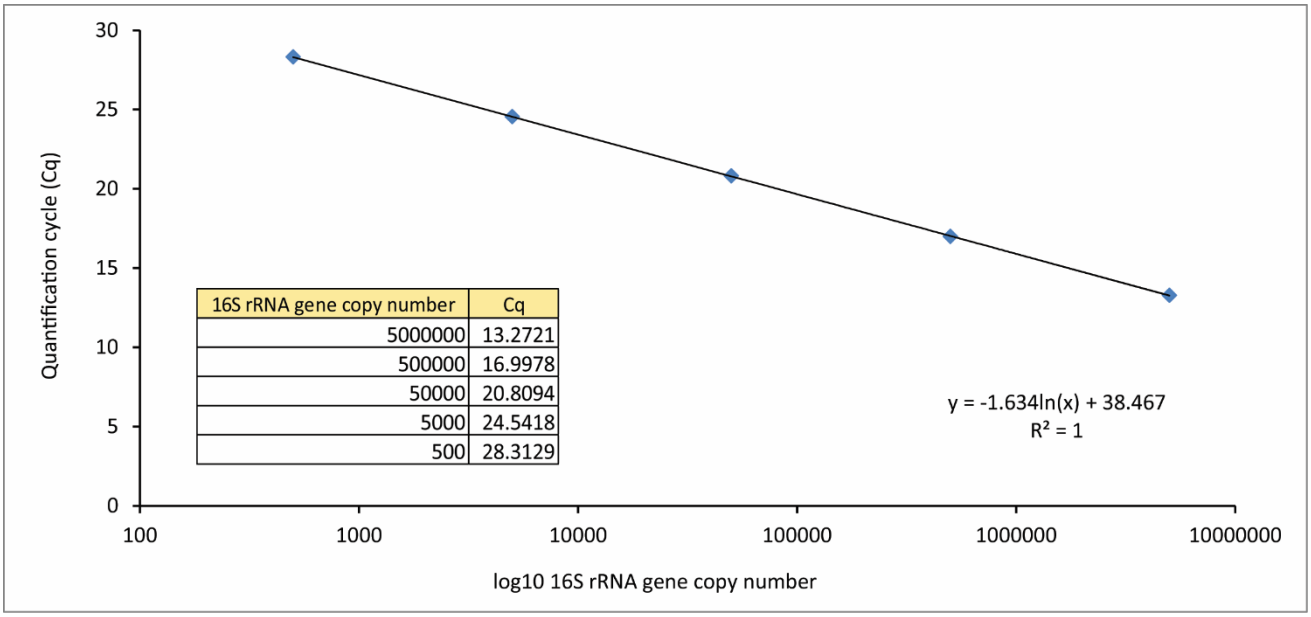
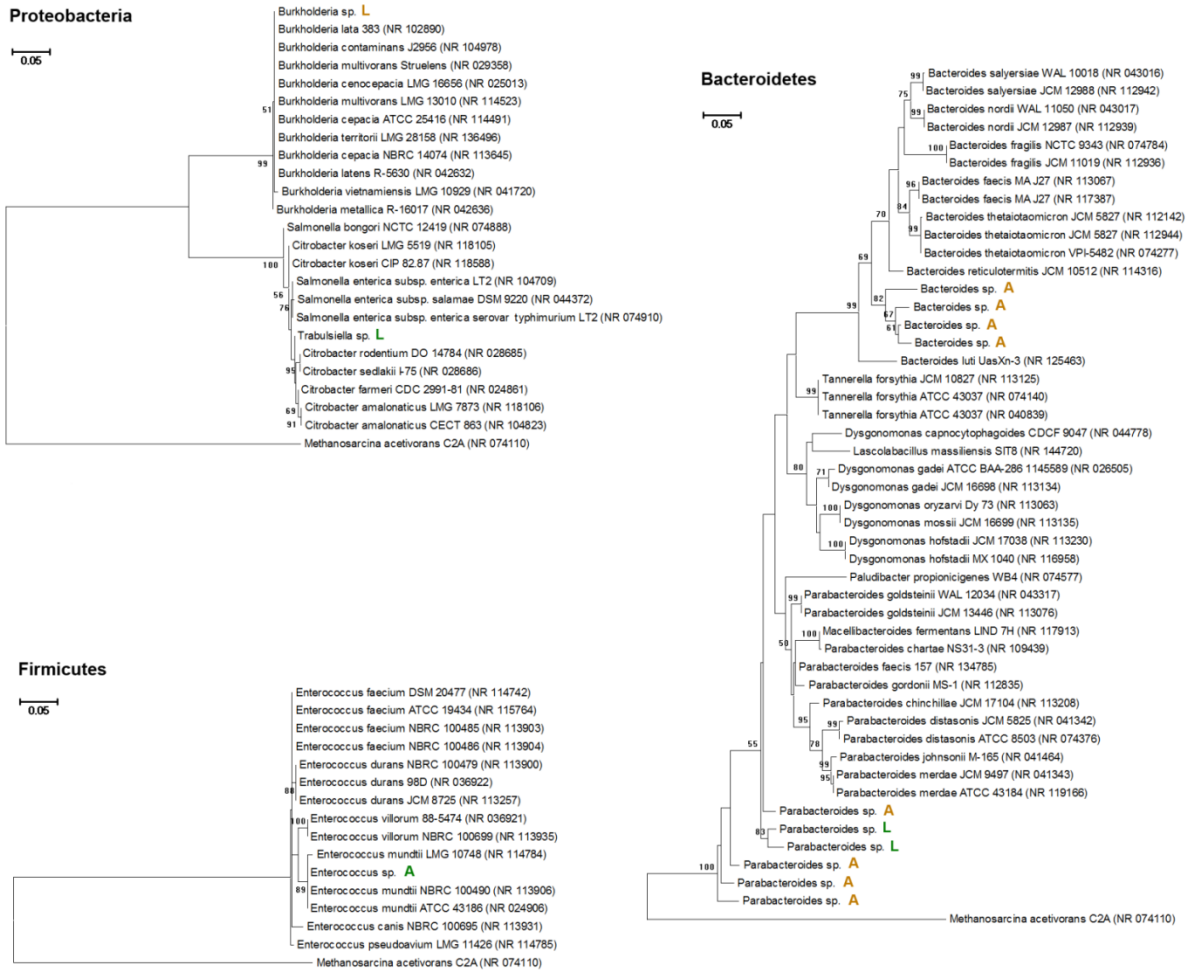
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

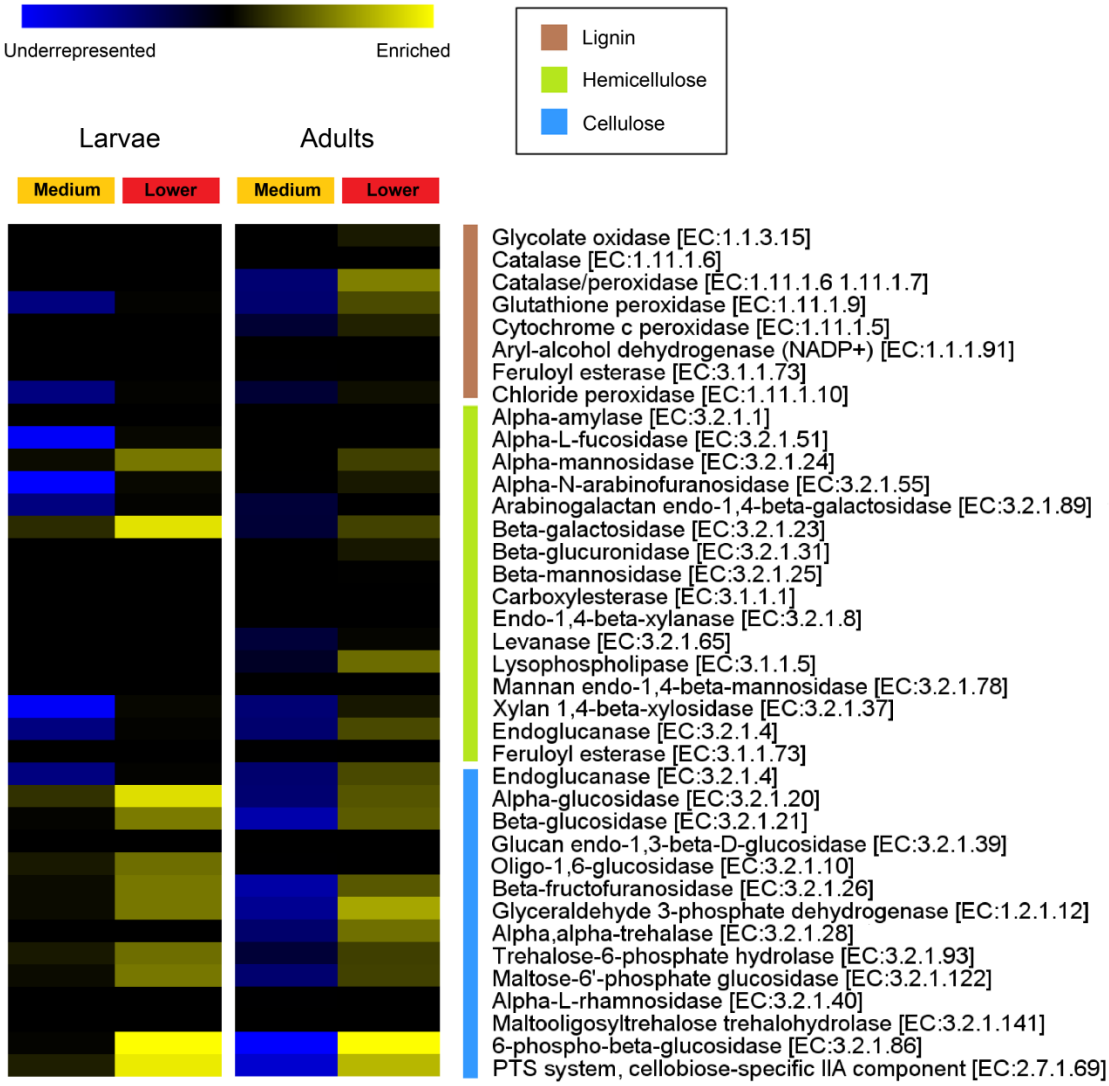


16S rRNA gene copy number	Cq
5000000	13.2721
500000	16.9978
50000	20.8094
5000	24.5418
500	28.3129

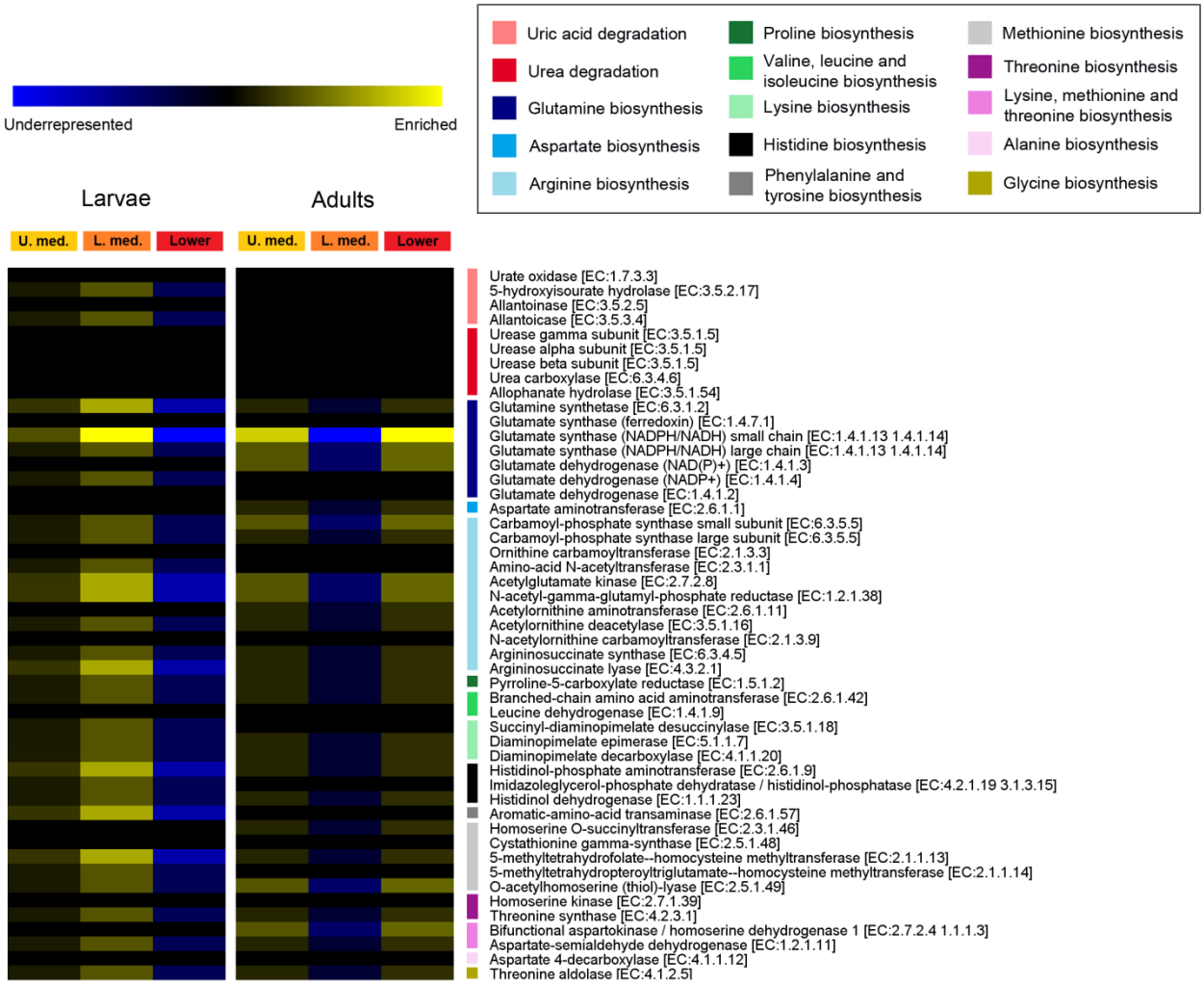
Supplementary Figure 1. qPCR standard curve constructed using *E.coli* genomic DNA, relating quantitative cycle values to $\log_{10}$ transformed 16S rRNA gene copy numbers.



Supplementary Figure 2. Maximum likelihood trees relating 16S rDNA sequences of the identified bacterial genera actively involved in the processing of urea and cellulose in *M. hippocastani* gut with their closest BLAST hits in the NCBI database. Active genera detected in the present study are indicated with colored letters A, when coming from adult gut, L, when coming from larval gut. Green indicates that the labeling was done with cellulose, orange indicates that the labeling was done with urea. Reference sequences were downloaded from GenBank (accession numbers are in parentheses). *Methanosarcina acetivorans* (NR 074110) was used as an outgroup. Bootstrap values (in percentages) are based on 1000 replications. Bar represents 5% sequence divergence.



Supplementary Figure 3. Heatmaps generated after PICRUSt prediction, displaying the enrichment of KEGG Orthologs involved in lignocellulose degradation in the ^{13}C medium and lower fractions compared to the same fractions in the ^{12}C gradient. The enrichment or underrepresentation of a certain ortholog is an indication of its presence or absence among the isotopically labeled bacterial families (Lachnospiraceae and Enterococcaceae in larvae, Enterobacteriaceae in adults). Blue represents underrepresentation, yellow represents enrichment.



Supplementary Figure 4. Heatmaps generated after PICRUSt prediction, displaying the enrichment of KEGG Orthologs involved in nitrogenous waste degradation and the synthesis of amino acids in the ^{15}N medium and lower fractions compared to the same fractions in the ^{14}N gradient. The enrichment or underrepresentation of a certain ortholog is an indication of its presence or absence among the isotopically labeled bacterial families (Burkholderiaceae in larvae, Porphyromonadaceae in adults). Blue represents underrepresentation, yellow represents enrichment.

Supplementary Table 1. Weighted NSTI values (average branch length (in 16S rRNA substitutions/site) that separates each OTU from a reference bacterial genome, weighted by the abundance of that OTU in the sample) of PICRUSt predicted metagenomes.

Gradient fraction	Weighted NSTI value	Gradient fraction	Weighted NSTI value
Larvae ^{12}C Medium	0.053388003573	Larvae ^{14}N Upper medium	0.05254
Larvae ^{12}C Lower	0.0541966375121	Larvae ^{14}N Lower medium	0.0523413375461
Larvae ^{13}C Medium	0.0605356305385	Larvae ^{14}N Lower	0.0523634453782
Larvae ^{13}C Lower	0.0600383912423	Larvae ^{15}N Upper medium	0.052345037037
Adults ^{12}C Medium	0.0501140916073	Larvae ^{15}N Lower medium	0.0523391317652
Adults ^{12}C Lower	0.075265354104	Larvae ^{15}N Lower	0.0523584971098
Adults ^{13}C Medium	0.0851468138346	Adults ^{14}N Upper medium	0.00976
Adults ^{13}C Lower	0.0671953641744	Adults ^{14}N Lower medium	0.00976
		Adults ^{14}N Lower	0.00976
		Adults ^{15}N Upper medium	0.00976
		Adults ^{15}N Lower medium	0.00976
		Adults ^{15}N Lower	0.00976

3.2 Article II

Draft Genome Sequence of *Enterococcus mundtii* SL 16, an Indigenous gut Bacterium of the Polyphagous Pest *Spodoptera littoralis*

Bosheng Chen, Chao Sun, Xili Liang, Xingmeng Lu, Qikang Gao, Pol Alonso-Pernas, Beng-Soon Teh, Alexey L. Novoselov, Wilhelm Boland and Yongqi Shao

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Draft Genome Sequence of *Enterococcus mundtii* SL 16, an Indigenous Gut Bacterium of the Polyphagous Pest *Spodoptera littoralis*

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Keywords: ***Enterococcus mundtii***, **genome sequencing, symbiosis,** ***Spodoptera littoralis***, **intestinal tract**

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INTRODUCTION

Insects are the most abundant and diverse animal class on Earth, and they are associated with an amazing variety of symbiotic microorganisms, which participate in many relationships with the hosts (Douglas, 2015). For example, the fungal symbiont (*Leucoagaricus gongylophorus*) of leaf-cutting ants produces diverse enzymes for the degradation of plant material (Kooij et al., 2016). Similarly, *Bacillus pumilus* isolated from the gut of wood boring *Mesomorphus* sp. (Coleoptera: Tenebrionidae) exhibits significant cellulolytic and xylose isomerase activities (Balsingh et al., 2016).

The Lepidoptera, including moths and butterflies, is one of the most widespread and widely recognizable insect orders in the world. Although butterflies and moths play an important role in the natural ecosystem as pollinators and as food in the food chain, their leaf-chewing larvae are often problematic in agriculture, as their main source of food is live plants (Mithöfer and Boland, 2012). The leafworm *Spodoptera littoralis* (Lepidoptera: Noctuidae) is a highly polyphagous lepidopteran pest found worldwide and also an important model system used in a variety of biological research. Recent extensive surveys of its microbiome reveal that *Enterococcus mundtii* is one of the predominant gut microorganisms of *S. littoralis* and present at high frequency (Tang et al., 2012; Chen et al., 2016; Teh et al., 2016). Particularly, a stable isotope labeling-based approach suggested that this phylotype was also highly metabolically active inside the host across life history of *S. littoralis*, indicating the significant role played by *E. mundtii* in host biology (Shao et al., 2014). Therefore, the symbiotic *E. mundtii* probably constitutes a key factor for the success of this generalist herbivore in adapting to different environments and food sources. The aim of this study was to produce a genome sequence of the strain SL 16, which would assist in understanding of the

coevolution of the microbe and the insect host. The dataset has been submitted to NCBI Whole Genome Shotgun (WGS) projects and is reported here, providing an overview of the genome sequence and relevant features of gut symbiotic *E. mundtii*.

MATERIALS AND METHODS

Isolation of the Bacterial Strain

E. mundtii strain SL 16 was isolated from the mature 5th instar larva using standard microbiology methods. Briefly, the normal larvae were washed and sedated on ice for at least 1 h to anesthetize them. Then the whole gut sections were dissected from larvae using a fine Vannas scissor and forceps under a binocular microscope (Shao et al., 2013). The fresh gut tissues were put into phosphate buffered saline (PBS: 137 mM NaCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and 2.7 mM KCl) and homogenized by hand with a sterile pestle. Bacterial isolates were made by plating the homogenized gut tissues on the *Enterococcus* Selective Agar (45183, Fluka). After incubation for 24 h at 30°C, the growing bacterial colonies were sub-cultured twice on the same agar medium. 2, 3, 5-Triphenyltetrazolium chloride (TTC) in the medium is reduced to insoluble formazan inside the bacterial cells, which gives pink or red coloration to enterococcal colonies. These purified enterococcal colonies were tested for key phenotypic traits including carbohydrate fermentation capability, motility, and pigment production as previously described (Manero and Blanch, 1999). Furthermore, the taxonomy was validated by colony PCR and sequencing of the amplified 16S rRNA gene. The representative *E. mundtii*

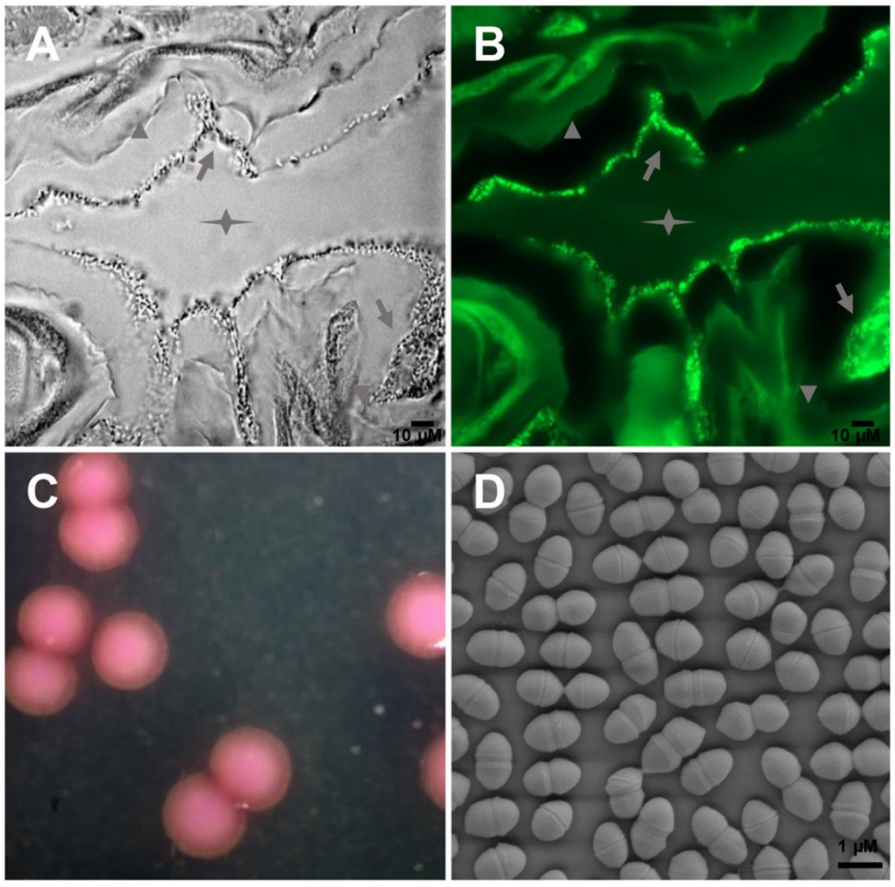

FIGURE 1 | Images of *E. mundtii* from *S. littoralis* reveal bacterial gut localization and phenotypic characteristics. (A) Phase-contrast micrograph and **(B)** FISH with a FITC-labeled Enterococcus-specific probe (green) show a high density of bacterial cells adhere on the mucus layer lining the gut epithelium. Star indicates the gut lumen, arrowhead indicates the gut epithelium tissue, and arrow indicates bacteria. **(C)** Photomicrograph of source organism on Enterococcus selective agar. 2, 3, 5-Triphenyltetrazolium chloride (TTC) in the agar is reduced to insoluble formazan inside the bacterial cells, which gives pink or red coloration to colonies. **(D)** Scanning electron micrograph of *E. mundtii* SL 16, showing cell division.

isolate, designated strain SL 16, was selected for this WGS project.

Fluorescence in situ Hybridization (FISH) was applied to localize the dominant enterococci as previously described (Shao et al., 2014). Shortly, FISH was performed on 5 μm thin cross sections of the cold polymerizing resin (Technovit 8100, Heraeus Kulzer GmbH, Wehrheim, Germany) embedded gut tissue. The specificity of probes was tested and hybridization condition was achieved as described (Tang et al., 2012). The sample was hybridized with 1.5 mM FITC-labeled *Enterococcus*-specific probe in hybridization buffer containing 900 mM NaCl, 20 mM Tris-HCl (pH 8.0), 20% formamide, 1% SDS. And images were taken with an Axio Imager Z1 microscope (Carl Zeiss, Jena, Germany). For scanning electron microscopy (SEM), cells were fixed in paraformaldehyde (1%), and glutaraldehyde (0.25%), dehydrated by ascending alcohol series and dried. After coating samples with gold, scanning electron micrographs were taken with a LEO 1525 instrument (Carl Zeiss, Jena, Germany).

Genomic DNA Isolation, Library Preparation and Sequencing

The genomic DNA was extracted from the cultured bacterium according to Pospiech and Neumann (1995). DNA quality was examined by 1% agarose gel electrophoresis and quantified using a NanoDrop™ spectrophotometer. The DNA library was constructed using the TruSeq™ DNA Sample Preparation Kit (Illumina Inc., San Diego, CA), and 5 μg of pure genomic DNA was prepared for a standard Illumina shotgun library construction. Briefly, genomic DNA was first sheared to a size ranging between 400 and 500 bp using the Covaris M220 per the manufacturer's recommendations. The fragmented DNA sample was end-repaired, dA-tailed, and ligated to multiplex adapters according to the manufacturer's instructions. The ligated products were purified and further enriched using PCR. The quality of the final amplified libraries were checked by running an aliquot (1 μL) on a high-sensitivity Bioanalyzer 2100 DNA Chip (Agilent Technologies). Paired-end sequencing was performed by using an Illumina MiSeq platform (Illumina Inc., San Diego, CA) at Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China) according to the manufacturer's instructions (Zhang et al., 2016).

Preprocessing and Genome Assembly

The quality of sequence reads was evaluated using the FastQC tool as previously described (Balsingh et al., 2016). Reads with >10% Ns and/or 25–35 bases of low quality (≤Q20) were filtered out, and adapter and duplication contamination were removed as well as read ends were trimmed off. The filtered reads were assembled with Short Oligonucleotide Analysis Package (SOAP) *de novo* version 2.04 using a range of *k*-mer sizes (Li et al., 2009). Then GapCloser version 1.12 was used to close any internal gaps in the optimal scaffolded assembly. Repeats were predicted by RepeatMasker and Tandem Repeats Finder (TRF) tools (Rédou et al., 2016). Barrnap version 0.4.2 and tRNAscan-SE version 1.3.1 were employed to predict rRNAs and tRNAs respectively. The genome was annotated using Glimmer version 3.02 (Xu et al., 2014). The Clusters of Orthologous Groups of proteins (COG) categories were assigned to the SL 16 genome annotation using blastp (BLAST 2.2.28+) against the COG genes collection (Von Mering et al., 2005). The translations of the identified coding sequences (CDSs) were also used to search against the Protein family (Pfam) database with *E*-value cut-off of 1-e5. The metabolic pathway analysis was constructed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2014).

INTERPRETATION OF DATA SET

Whole Genome Sequencing of *E. mundtii* SL 16

Large amounts of *E. mundtii* closely adhere to the mucosal layer of *S. littoralis* gut epithelium, where they form a biofilm-like structure (**Figures 1A,B**). Strain SL 16 displays characteristic phenotypes of *E. mundtii*. It grows well on Slanetz and Bartley medium (Slanetz and Bartley, 1957), producing smooth, circular, glistening colonies (**Figure 1C**). The bacterial cells are 0.5–1.0 μm in diameter, and occur in the form of pairs (**Figure 1D**). Strain SL 16 could utilize various carbon sources, including xylose, cellobiose, and sucrose (**Table 1**).

Sequencing the genome of *E. mundtii* SL 16 produced a raw data set of 1,764,821,160 total bases. During the quality control, Illumina PCR adapter reads and low-quality reads were removed, and a total of 3,469,570 mate-pair reads (total bases 1,698,525,052 bp) were retained. The cleaned sequence reads were assembled with a *k*-mer setting of 125, which was determined by the optimal assembly result. The resulting genome sequence has an estimated size of 3,296,585 bp and a G+C content of 38.36%. 43,977 bp were repeats as predicted by RepeatMasker and TRF tools, which constituted 1.33% of the entire assembled genome.

TABLE 1 | *E. mundtii* SL 16 genome resources and characteristics.

	Name	Genome resources/characteristics
1	NCBI Bioproject ID	PRJNA337899
2	NCBI Biosample ID	SAMN05513637
3	NCBI Genome Accession Number	MCRG00000000
4	Sequence type	Illumina Miseq
5	Total number of Reads	3,515,580
6	Overall coverage	>100x
7	Estimated genome size (bp)	3,296,585
8	GC content (%)	38.36
9	Average of gene length (bp)	889
10	Protein coding genes	2939
11	tRNA coding genes	59
12	Motility	Non-motile
13	Cellobiose metabolism	Positive
14	Xylose metabolism	Positive
15	Arabinose metabolism	Positive
16	Sucrose	Positive

A total of 3125 genes with sequence length of 2,780,928 bp were predicted, which account for 84.4% of the genome, and 59 tRNA genes were identified by tRNAscan-SE. CDSs were searched against the NR, GO, string, Swiss-Prot, COG, and KEGG databases to analyze gene functions and metabolic pathways. In all, 1493 CDSs were assigned to COG families and 1411 CDSs were included in 154 pathways. Several physiological traits that may explain the successful adaptation of this bacterium to the environment of the gut have been found. In particular, a large amount of the coding capacity encountered in the genome of SL 16 (almost 12%) is dedicated to genes assigned to functions related to carbohydrate transport and metabolism, which matches well with the observed physiological characteristics of this strain (**Table 1**). This feature is shared with other colonic inhabitants, such as *Bacteroides fragilis* (Flint et al., 2008), and reflects the ecological niche of the organism presented inside a herbivore gut. The genome encodes several ABC-type sugar transporters, sugar-binding proteins, and a rich suite of glycosyl hydrolases, such as β-N-acetylhexosaminidase, α-galactosidase, β-glucosidase, β-galactosidase, and α-glucosidase. Moreover, the pyruvate dissipation pathways predicted for SL 16 include the capacity to produce L-lactate and several other fermentation metabolites, like short-chain fatty acids formate and acetate. This metabolic flexibility is expected to aid in efficient digestion and conversion of plant saccharides, thus promoting host development.

In conclusion, here we report a 3.30 Mbp draft genome sequence of *E. mundtii* strain SL 16, isolated from the generalist herbivore *S. littoralis*. The final *de novo* assembly is based on 1765 Mbp of Illumina data which provides an average coverage of 535 ×. Analysis of the genome shows high correlation between the genotypes and the phenotypes.

Direct Link to Deposited Data and Information to Users

The dataset submitted to NCBI include the assembled consensus sequence of *E. mundtii* SL 16 in Fasta format. The genome sequence can be accessed at DDBJ/EMBL/GenBank under the accession no. MCRG00000000. This paper describes the first version of the genome (https://www.ncbi.nlm.nih.gov/nuccore/MCRG00000000).

AUTHOR CONTRIBUTIONS

Work was planned by YS and WB, and executed jointly by BC and CS. XLi and BT were associated with isolation of the bacterium. AN and PA performed bioinformatics analyses. QG and XLu contributed to the DNA sequencing.

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3.3 Article III

Bacterial Community and PHB-Accumulating Bacteria Associated with the Wall and Specialized Niches of the Hindgut of the Forest Cockchafer (*Melolontha hippocastani*)

Pol Alonso-Pernas, Erika Arias-Cordero, Alexey Novoselov, Christina Ebert, Jürgen Rybak, Martin Kaltenpoth, Martin Westermann, Ute Neugebauer and Wilhelm Boland

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Bacterial Community and PHB-Accumulating Bacteria Associated with the Wall and Specialized Niches of the Hindgut of the Forest Cockchafer (*Melolontha hippocastani*)

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A characterization of the bacterial community of the hindgut wall of two larval and the adult stages of the forest cockchafer (*Melolontha hippocastani*) was carried out using amplicon sequencing of the 16S rRNA gene fragment. We found that, in second-instar larvae, Caulobacteraceae and Pseudomonadaceae showed the highest relative abundances, while in third-instar larvae, the dominant families were Porphyromonadaceae and Bacteroidales-related. In adults, an increase of the relative abundance of Bacteroidetes, Proteobacteria (γ- and δ- classes) and the family Enterococcaceae (Firmicutes) was observed. This suggests that the composition of the hindgut wall community may depend on the insect's life stage. Additionally, specialized bacterial niches hitherto very poorly described in the literature were spotted at both sides of the distal part of the hindgut chamber. We named these structures "pockets." Amplicon sequencing of the 16S rRNA gene fragment revealed that the pockets contained a different bacterial community than the surrounding hindgut wall, dominated by Alcaligenaceae and Micrococcaceae-related families. Poly-β-hydroxybutyrate (PHB) accumulation in the pocket was suggested in isolated *Achromobacter* sp. by Nile Blue staining, and confirmed by gas chromatography–mass spectrometry analysis (GC-MS) on cultured bacterial mass and whole pocket tissue. Raman micro-spectroscopy allowed to visualize the spatial distribution of PHB accumulating bacteria within the pocket tissue. The presence of this polymer might play a role in the colonization of these specialized niches.

Keywords: hindgut, *Melolontha hippocastani*, gut bacteria, poly-β-hydroxybutyrate, PHB, *Achromobacter*, Raman microscopy

INTRODUCTION

Bacteria not only thrive as free-living organisms in the environment, they also engage in complex symbiotic relationships with higher organisms (Wells and Varel, 2011). Insects, in particular, are associated with a large diversity of microorganisms that play important roles for their host's physiology, ecology, and evolution. The insect gut is colonized by a wide range of bacterial phylotypes that interact with the host and allow it to subsist on nutritionally imbalanced diets. The recycling of nitrogen, the provisioning of essential amino acids and cofactors, and the digesting of recalcitrant polymers in the host's diet are among the functions for which symbiotic microorganisms play an integral role (Potrikus and Breznak, 1981; Douglas, 2009; Watanabe and Tokuda, 2010), increasing the overall fitness of the insect host.

Typically, the insect gut is divided into three regions, i.e., foregut, midgut and hindgut. The symbiotic bacteria are either attached to the gut wall or colonize the gut as free-living organisms, usually mostly in the mid- and hindgut regions. The structure of these communities differs among insect species, influenced by the host's diet and taxon (Egert et al., 2003, 2005; Colman et al., 2012; Jones et al., 2013). In the Scarabaeidae family, the hindgut region is of special importance. It is anatomically modified to serve as fermentation chamber. This chamber, in addition to its original function, namely, absorbing water and salts from the gut content, is also devoted to aiding digestion, probably with the help of the fermentative bacteria that colonize it (Egert et al., 2005; Huang et al., 2010; Arias-Cordero et al., 2012; Engel and Moran, 2013). These microbial associates are transmitted either vertically, directly from mother to offspring, or horizontally, that is, being taken anew from the environment by each host generation (Bright and Bulgheresi, 2010). In horizontally transmitted symbiosis, the host usually ingests the symbiont along with unwanted microbes that may compete for the colonization of the gut. The selection of the right symbiont may depend on its phenotypic traits. Kim et al. (2013) showed that poly-β-hydroxybutyrate (PHB) accumulation by the symbiont is crucial for the maintenance of host–microbe relationship.

In this study, we investigate the forest cockchafer (*Melolontha hippocastani*). This scarabaeid constitutes an interesting model due to its particular life cycle, consisting in two well-differentiated stages: the rhizophagous larvae spend up to 4 years underground, while the adults, after pupation, emerge from the soil and shift to a diet based exclusively on foliage. To date, there is a lack of comparative studies on the variation of the gut bacterial community associated with the transition from larva to adult. Only one study addressed this question, a study conducted by Arias-Cordero et al. (2012), focused on the midgut of *M. hippocastani*. Surprisingly, they found a group of bacterial phylotypes that seems to always be stable. This core community is maintained through metamorphosis and is unaffected by the radical change of the host diet from roots to leaves, when the shift occurs from a below-ground (larval) to an above-ground (adult) stage (Arias-Cordero et al., 2012).

In view of this unexpected stability of the gut microbial community, we considered appropiate to characterize the bacterial communities inhabiting the hindgut wall of both below- and above-ground stages of the forest cockchafer, thus complementing the above-mentioned midgut-based study (Arias-Cordero et al., 2012). We put our focus on the hindgut wall itself, and also on particular bacterial niches attached and connected to it, at both sides of the distal part of the larval hindgut. These small structures, called from now on "pockets," have been hitherto only once described in the literature (Wildbolz, 1954). They consist of several tubular poles connected to the hindgut chamber, which contain bacterial phylotypes that are minor or not detected in the hindgut wall. We detected the presence of PHB within the pockets, and *Achromobacter* sp., one of the major pocket bacterial species, is able to accumulate PHB in pure culture. This suggests that some of the pocket symbionts may be horizontally transmitted, as previous studies found this type of inclusions in symbiotic *Burkholderia* of environmental origin harbored in the midgut crypts of the midgut of *Riptortus pedestris* (Kim et al., 2013). The question of whether PHB plays a role in host nutrition remains unknown.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

Second-instar (L2) and third-instar (L3) larvae of *M. hippocastani* and actively flying adults were collected in forests of red oak in Mannheim (49°29′20″N 8°28′9″E), and Graben-Neudorf (49°9′55″N 8°29′21″E), respectively, between December 2010 and May 2014. Beetles were collected at the same sites. The insects were transported alive in boxes with soil or tree leaves. Before dissection, the insects were kept at −20°C for 20 min to kill them, and then rinsed three times alternately with sterile distilled water and 70% ethanol. Dissection was performed on ice in a phosphate-buffered saline (PBS) solution. Hindguts, as shown between dotted lines in **Figure 1D** (top for larva and bottom for adult), were excised, cut open, and carefully washed three times with sterile PBS in order to remove any unattached bacteria. The pockets were separated from the hindgut wall, and as much of the surrounding epithelium was removed as possible. Samples were stored at −20°C before DNA extraction. The day of the extraction, frozen samples were thawed on ice and dried at 45°C for 90 min in a Speedvac (Concentrator 5301, Eppendorf), then crushed in a 1.5 ml tube with a sterile pestle. For 454-pyrosequencing, DNA extractions of the tissue were carried out using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) according to the protocol provided by the manufacturer. Final DNA concentrations were determined using a Nanovue device (GE Healthcare, Little Chalfont, UK). In order to test for the quality of the extracted DNA and confirm the presence of DNA from bacteria, a diagnostic PCR reaction was carried out as described (Arias-Cordero et al., 2012).

Transmission Electron Microscopy (TEM)

Dissected hindguts and pockets of larvae were fixed in a solution of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M

sodium cacodylate buffer (pH 7.2). Immediately afterward, the tissue was transferred to the same solution for overnight fixation. Next day, the fixative was removed, and the tissue was post fixed with 1% osmium tetroxide in cacodylate buffer for 2 h. During the following ascending ethanol series samples were stained with 2% uranyl acetate. The samples were embedded in Araldite CY212 epoxy resin (Agar Scientific Ltd, Stansted, United Kingdom) according to manufacturer's instruction. Semi-thin sections (1 μm thickness) were stained with Richardson's methylene blue in order to localize the right position for the examination. Hindgut areas were further trimmed down to 500 μ × 500 μm. Ultra-thin sections of 80 nm thickness were cut using an ultramicrotome Ultracut E (Reichert–Jung, Vienna, Austria) and mounted on Formvar-carbon coated grids (100 meshes, Quantifoil GmbH, Großlöbichau, Germany). Finally, sections were contrasted with lead citrate for 4 min and analyzed in a transmission electron microscope EM900 (Zeiss AG, Oberkochen, Germany).

Light Microscopy, Richardson Staining, and Autofluorescence Visualization

In all cases the tissue was fixed as described above for transmission electron microscopy (TEM). The tissues employed were larvae hindgut walls and pockets. For the Richardson staining, semi-thin sections of 0.3–0.6 μm (embedded as for TEM) were immersed in a 60°C staining solution for 3–5 min. Afterward, the tissue was washed twice with sterile water. Finally, the sections were placed on a glass slide, dried and mounted for microscopic observation. For the autofluorescence visualization, a excised complete hindgut pocket was placed onto a glass slide and covered with PBS. Visualization was carried out using a LeicaTCS-SP2 confocal microscope using a 10× dry or 40× oil Leica objective (HC PL APO 10×/0.4, Leica, Bensheim, Germany) in both cases. For autofluorescence, laser line employed was 488 nm.

Bacterial Tag-Encoded FLX Amplicon Pyrosequencing (bTEFAP) and Data Analysis

For pyrosequencing, a sample was composed of the extracted DNA of six insects collected during the same year, pooled together in equal amounts for a single run. A total of four samples were sequenced (L2 pocket, L2 hindgut wall, L3 hindgut wall, and adult hindgut wall). DNA was sent to an external service provider (Research and Testing Laboratories, Lubbock, TX, USA) for bTEFAP with 16S rRNA primers Gray28F (5′-GAGTTTGATCNTGGCTCA-3′) and Gray519R (5′-GTNTTACNGCGGCKGCTG -3′) (Ishak et al., 2011). A sequencing library was generated through one-step PCR with 30 cycles, using a mixture of HotStar and HotStar HiFidelity *Taq* polymerases (Qiagen, Hilden, Germany). Sequencing extended from Gray28F, using a Roche 454 FLX instrument with Titanium reagents and procedures at Research and Testing Laboratory (RTL, Lubbock, TX, USA[1]). Quality control and analysis of 454 reads, including calculation of rarefaction curves and community richness and diversity indexes, was done in QIIME version 1.8.0 (Caporaso et al., 2011). Low-quality ends of the sequences were trimmed with a sliding window size of 50 and an average quality cut-off of 25. Subsequently, all low-quality reads (quality cut-off = 25) and sequences <200 bp were removed, and the remaining reads were denoised using the "denoiser" algorithm as implemented in QIIME (Reeder and Knight, 2010). Denoised high-quality reads were clustered into operational taxonomic units (OTUs) using a multiple OTU picking strategy with cdhit (Li and Godzik, 2006) and uclust (Edgar, 2010), with 97% similarity cut-offs, respectively. For each OTU, the most abundant sequence was chosen as a representative sequence and aligned to the Greengenes core set[2] using PyNast (Caporaso et al., 2010). RDP classifier was used for taxonomy assignment (Wang et al., 2007). An OTU table was generated describing the occurrence of bacterial phylotypes within the samples.

qPCR Analysis of Pocket and Hindgut Wall Tissue

For the quantitative real-time PCR (qPCR) analysis, third-instar larvae were used. A sample was composed of the pooled DNA from hindgut wall, or pockets, of three different larval individuals. Three samples from each tissue (hindgut wall and pockets) were considered, and each one was analyzed per triplicate. Specific primers were designed using Geneious 6.0.5[3] for the five most consistently found bacterial taxa in the pocket (*Achromobacter*, *Citrobacter*, *Bosea*, *Brevundimonas*, and *Pseudomonas*), based on the alignment of the representative set of sequence data for all OTUs available from the 454-pyrosequencing. PCR conditions for each primer pair were optimized using gradient PCRs (Salem et al., 2013). Their specificity was verified *in silico* against the SILVA ribosomal RNA database[4] and *in vitro* by sequencing. Briefly, PCR products from pocket DNA were analyzed on 1% agarose gels (150 V, 30 min). The products were purified from the gel with Invisorb Fragment CleanUp kit (Stratec Molecular, Berlin, Germany) and cloned in pCR 2.1 vector using the Original TA Cloning kit (Invitrogen, Carlsbad, CA, USA). Ninety clones with positive inserts were selected according to the manufacturer's protocol and sequenced on a 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA) with BD 3.1 chemistry. If the sequence matched the expected OTU, the primer pair was assumed to specifically amplify the target OTU within the gut and pocket. The sequences of the primers are listed in Supplementary Table S2. Quantitative PCRs for individual bacterial taxa were performed on a CFX96 Real Time System (Bio-Rad, Munich, Germany), in final reaction volumes of 10 μL containing 1 μL of template DNA (usually a 1:10 dilution of the original DNA extract), 0.6 μL of each primer (10 pM) and 5 μL of SYBR Green Mix (Rotor-Gene SYBR Green kit, Qiagen, Hilden, Germany). Standard curves were established using 10^{-6}–10^{-2} ng of specific

[1] www.researchandtesting.com/

[2] http://greengenes.lbl.gov/

[3] http://www.geneious.com

[4] http://www.arb-silva.de

PCR product as templates for the qPCR. A NanoDrop ND-1000 spectrophotometer (Peqlab Biotechnology Limited, Darmstadt, Germany) was used to measure template DNA concentration for the standard curve. Five different replicates of the standard concentrations for each bacterial taxon were used to calculate a correction factor and determine equitation parameters. PCR conditions were as follows: 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing for 30 s and elongation at 72°C for 10 s. Then, a melting curve analysis was performed to ensure that amplicons were the same across samples for each primer assay, by increasing the temperature from 65 to 95°C within 5 min. The annealing temperature was specific for each primer pair: for *Achromobacter* and *Citrobacter*, 60°C; for *Bosea*, 63°C; for *Brevundimonas*, 55°C; for *Pseudomonas*, 68°C. Based on the standard curves, the 16S copy number could be calculated for each individual sample from the qPCR threshold values (Ct) by the absolute quantification (Lee et al., 2006, 2008), taking the dilution factor and the absolute volume of DNA extract into account. The quantitative differences in the microbial community abundances of the pocket were tested using SPSS 17.0 (Tukey HSD test, confidence interval of 0.05).

Isolation and Identification of Pocket Bacteria

Four second-instar pockets from different larvae were dissected as mentioned above and incubated together in a 0.8% NaOCl aqueous solution for 3 min on ice for surface sterilization. Then, the tissue was transferred in Ringer+ppi buffer (Cazemier et al., 1997) and sonicated using a Sonorex Super RK 102h sonicator (Bandelin, Germany) for 7 min at RT. After sonication, the tubes were incubated 15 min on ice and gently tapped from time to time. Ten-fold dilutions of the supernatant were plated on LB agar (Carl Roth, Germany) and ATCC agar in order to enrich for *Achromobacter* sp. The ATCC agar contained (per liter): 7.32 g K_2HPO_4, 4.6 g ammonium tartrate, 1.09 g KH_2PO_4, 0.04 g $MgSO_4$ $7H_2O$, 0.04 g $FeSO_4$ $7H_2O$, 0.014 g $CaCl_2$ $2H_2O$, and 35 g agar. Plates were incubated at 30°C for 48 h. Morphologically different colonies were subcultured three times before identification. Colony PCR targeting the small ribosomal subunit gene was performed on a GeneAmp 9700 Thermocycler (Applied BioSystems) using the general bacterial primers 27f and 1492r (Arias-Cordero et al., 2012). The 50 μL reaction mixture contained 1x buffer, 1.5 mM $MgCl_2$, 10 mM of the four deoxynucleotide triphosphates (dNTPs), 2.5 U Taq DNA polymerase (Invitrogen) and 0.5 mM of each primer. The PCR program was as follows: initial denaturation at 94°C for 3 min followed by 32 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 30 s and elongation at 72°C for 1 min, and a final elongation step at 72°C for 10 min. Amplicon size was confirmed in a 1% agarose gel; then the PCR product was purified using the Invisorb Fragment CleanUp kit (STRATEC Molecular GmbH, Berlin, Germany). Sequencing was performed at Macrogen Europe (Amsterdam, The Netherlands), and the taxonomy of resulting sequences was assigned using Basic Local Alignment Search Tool (BLAST) (Tatusova and Madden, 1999).

Metabolic Testing of Bacterial Isolates

Nile Blue agar was prepared as described (Luellen and Schroth, 1994). A representative of each bacterial isolate was plated and incubated for 48 to 72 h at 30°C. The plates were then viewed under UV light to detect putative PHB production based on the fluorescence of the colonies. Nitrate reduction test was purchased from Sigma and conducted following the instructions provided by the manufacturer. A representative of each bacterial isolate was inoculated at high density, and tubes were sealed with liquid paraffin to create oxygen-poor conditions and incubated at 30°C up to 5 days.

Gas Chromatography – Mass Spectrometry

Twenty-five third-instar larvae were dissected as described, and their 50 pockets were analyzed as one single sample. *Achromobacter* sp. isolated from the pocket was cultured for 3 days in PHB inducing broth at 30°C for 72 h. The composition of PHB inducing broth is the same as Nile Blue agar (Luellen and Schroth, 1994) without Nile Blue or agar. The bacterial mass was recovered by centrifugation and washed twice with sterile distilled water prior to drying [45°C for 90 min in a Speedvac (Concentrator 5301, Eppendorf)]. 5 mg (dry weight) of bacterial mass was used for the analysis. Poly[(*R*)-3-hydroxybutyric acid] standard was obtained from Sigma (Germany), and 1 mg was used for the analysis. Derivatization was performed as described (Riis and Mai, 1988), using methanol instead of propanol for the esterification. GC analysis was performed in a ThermoQuest, Finnigan Trace GC-MS 2000 series (Egelsbach, Germany), equipped with a fused-silica capillary Phenomenex ZB-5 column (15 m × 0.25 mm, film thickness 0.25 μm) with a split ratio of 10:1. Helium was used as carrier gas at a flow rate of 1.5 ml/min. The oven temperature was programmed as follows: the initial temperature of 60°C was held for 3 min, then increased to 230°C at 30°C/min and held for 2 min. The inlet temperature was 250°C and the injection volume 1 μL. Mass spectra were measured in electron impact (EI) at 70 eV under full scan mode (m/z 35–575). Acquired data were further processed using the software Xcalibur (Thermo Scientific). 3-hydroxybutyric acid methyl esters were identified by comparison of the mass spectrum and retention time with poly[(*R*)-3-hydroxybutyric acid] standard.

Raman Micro-Spectroscopy

One pocket was used for each Raman measurement. CaF_2 slides suitable for Raman spectroscopy were poly-L–lysine coated by being soaked overnight in 0.1% poly-L–lysine solution (Sigma) at 4°C prior to measurement. The pocket tissue was fixed overnight with 4% paraformaldehyde solution in 0.9% NaCl at 4°C. After fixation, the paraformaldehyde was removed and the tissue was washed three times for 10 min with 0.9% NaCl solution under mild agitation. Then the pocket tissue was embedded in a mounting medium for cryotomy, OCT compound (VWR Chemicals, Radnor, PA, USA) and sliced in 12-μm thick sections using a Microm HM 560 cryomicrotome (Thermo Scientific, Waltham, MA, USA). The tissue slices were put onto the

poly-L–lysine coated CaF_2 slide, washed carefully with 0.9% NaCl to remove the remains of the mounting medium and viewed under a bright-field microscope to check for the characteristic round-shaped cross-sections of the pocket poles. The Raman spectra were acquired with a confocal Raman microscope alpha 300R (WITec, Ulm, Germany) using a 532 nm Nd:YAG solid laser with a power of 15 mW for excitation. The samples were measured in 0.9% NaCl using a 60× water immersion objective with NA 1.0 (Nikon, Tokyo, Japan). Collection of backscattered photons occurred through a back-illuminated CCD camera (DV401-BV-352, Andor, Belfast, UK). For spectral grating, 600 lines/mm were used for 532 nm. A multimode fiber of 25 μm diameter served as pinhole for confocal imaging. The Raman spectra were recorded by using 1 s integration time. Characteristic spectra and compartments in the pocket poles were detected by analyzing the Raman scans with the N-FINDR unmixing algorithm (Winter, 1999; Hedegaard et al., 2011) using Matlab software (MathWorks). The PHB was detected by identifying specific peaks through comparison with measured reference spectrum of pure PHB compound.

Nucleotide Sequence Accession Numbers

The 16S RNA gene sequences obtained by colony PCR have been deposited at the NCBI GenBank under accession numbers from KY178280 to KY178284 (**Table 1**). Pyrosequencing data from L2 hindgut wall, adult hindgut wall, L2 pocket and L3 hindgut wall have been deposited under accession numbers SRR5059348, SRR5059349, SRR5059340, and SRR5059351, respectively.

RESULTS

Localization and Morphology of the Pockets

During the dissection of larval individuals (**Figure 1A**), two small structures ["pockets," colored either white or black (**Figures 1E,G**)] attached outside the terminal point of the hindgut chamber (**Figures 1B,C,D,E, 2A**) were spotted. The pockets have a diameter of around 500 μm, and showed high autofluorescence when illuminated with a 488 nm laser (**Figure 2B**). They are covered by a fine layer of muscle tissue (Supplementary Figure S1). Their anatomy is composed by poles connected to the hindgut lumen (Supplementary Figure S2). Further anatomical investigation by TEM revealed that each pole was surrounded by a thick acellular tissue layer (possibly mucous-like, **Figure 2C**). Additionally, it was observed that each pole was lined with large numbers of bacterial cells (**Figure 2C**). These cells showed a high number of cytoplasmatic inclusions (**Figure 2D**).

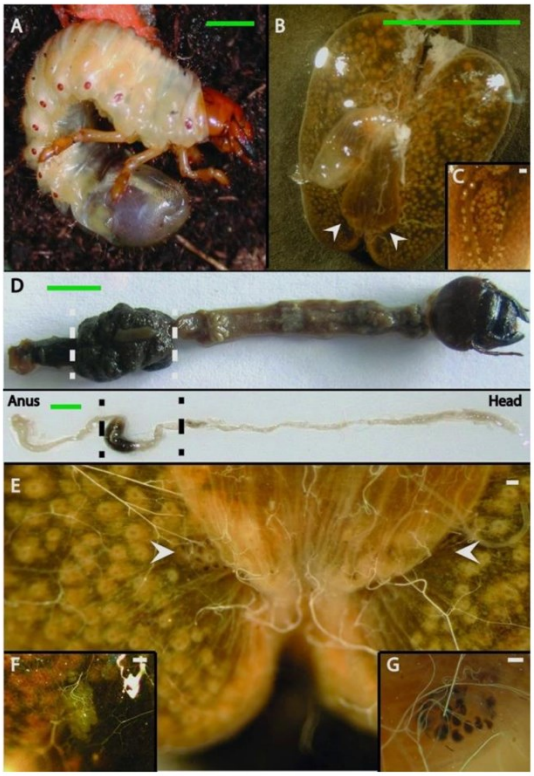

FIGURE 1 | Gut anatomy of larvae and adults of *Melolontha hippocastani*. **(A)** L3 larval instar living in the soil. **(B)** Hindgut fermentation chamber. White arrowheads point to the position of the pockets. **(C)** Close-up of a hindgut lobe. **(D)** Whole gut preparation of an L3 larval instar (top image) and an adult beetle (bottom image). The hindgut section used for microscopy and pyrosequencing is between the dashed lines. **(E)** The fermentation chamber and the pocket position (pointed with arrows). **(F)** Close-up of the *M. melolontha* pocket and **(G)** close-up of the *M. hippocastani* pocket. Scale bars: green 5 mm., white 100 μm.

TABLE 1 | Bacterial isolates from *Melolontha hippocastani*'s pockets with their metabolic capabilities.

Closest taxonomic affiliation	Identity percentage	Nile blue staining	Denitrification	Accession number
Citrobacter murliniae	99	Negative	Nitrate to nitrite	KY178281
Achromobacter marplatensis	99	Positive	Nitrate to nitrogen	KY178280
Ochrobactrum thiophenivorans	100	Negative	Negative	KY178282
Phyllobacterium myrsinacearum	99	Positive	NT	KY178283
Stenotrophomonas maltophilia	99	Positive	NT	KY178284

NT, not tested.

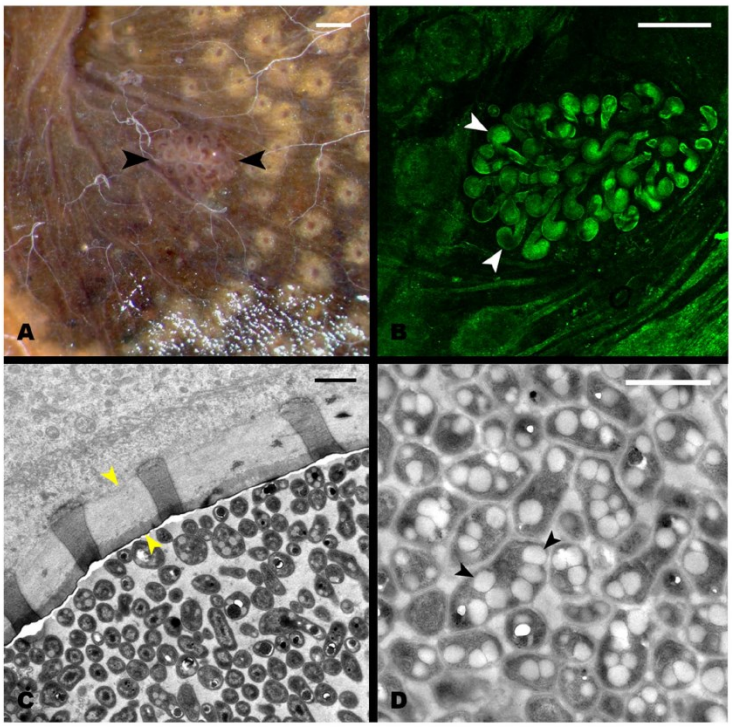

FIGURE 2 | Structure of the hindgut pocket. (A) Pocket attached to the hindgut external surface (black arrowheads). **(B)** Autofluorescence image of the pocket tissue using a 488 nm laser in a confocal microscope. White arrowheads point to pocket poles. **(C)** Transmission Electron microscopy (TEM) image of a cross-section of a pocket pole. The yellow arrowheads point to the acellular layer, possibly mucous-like, enveloping every pole. **(D)** TEM image of the dense bacterial population in the center of the pocket poles. Black arrowheads point to the PHB granules observed in the bacterial cytoplasm. Scale bars: **(A,B)** 100 μm, **(C,D)** 1 μm.

Pyrosequencing of the Bacterial Community from the Hindgut Wall of Adult Insects and Larvae, and Pockets

To establish the dynamics of the hindgut wall community across different host's life stages, the bacterial communities of the hindgut wall of L2 and L3 larvae and adults were compared. DNA from six different insects of each life stage was used, pooled together in a single pyrosequencing run. In the final output, 110,772 high quality reads were obtained (Supplementary Table S1). It was found that, in the L2 hindgut wall, the main bacterial phyla were Pseudomonadaceae, Caulobacteraceae and Micrococcaceae, while in L3 hindgut wall, those were Bacteroidetes phylum and Clostridia, with a large proportion of unknown bacteria. In the adults, an increase of the relative abundance of the Bacteroidales order, Proteobacteria (γ- and δ- classes) and the family Enterococcaceae (Firmicutes) was observed (**Figure 3**). Estimation of alpha-diversity in these samples was done using rarefaction methods, and richness and diversity indexes were also calculated (Supplementary Figure S3 and Table S1).

Amplicon sequencing revealed considerable differences in microbial communities between the L3 and L2 hindgut walls. In L2, approximately 47% of the sequences obtained belong to the family Pseudomonadaceae and 30% to the family Caulobacteraceae, taxa that were not detected in the L3 hindgut wall; the L3 hindgut wall, in turn, had families at high abundances which were not or only at low abundances detected in L2 (e.g., Porphyromonadaceae, Bacteroidales, and Ruminococcaceae) (**Figure 3**). This may reflect the changes that the bacterial community undergoes throughout the different stages of the insect's life, suggesting that the hindgut wall is a dynamic environment.

A pooled sample of DNA extracted from 12 excised pockets (from 6 L2 larvae) was also sequenced, in order to compare their bacterial communities with the surrounding hindgut wall. It was found that the main bacterial phyla of the pocket tissue were Actinobacteria and Proteobacteria (α- and β- classes). Within the β-Proteobacteria, *Achromobacter* sp., which accounted for 85% of sequences from the family Alcaligenaceae, was the genus with the overall highest relative abundance in the pockets. The classification at genus level of the family Micrococcaceae was not achieved. These two families were present in low abundance in the L2 and L3 hindgut wall, as well as in the hindgut wall of adult beetles (**Figure 3**).

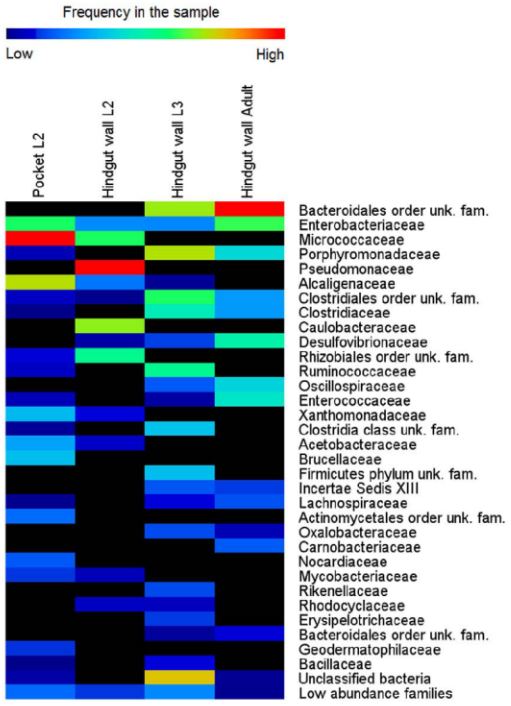

FIGURE 3 | Bacterial community composition in different life stages of *M. hippocastani*. Relative abundance of bacterial taxa, in percentage of total sample sequences, from 454 pyrosequencing data (110,772 sequences in total) is displayed as a heat map based on the log-transformed values. Warm colors indicate higher and cold colors lower abundances. Families with total relative abundance lower than 0.4% were considered "low abundance families" and listed in Supplementary Table S3. unk., unknown; fam., family.

Estimation of Absolute Abundances of Main Bacterial Genera in the Pockets and the Hindgut Wall

In order to compare the absolute abundances of key genera inhabiting the pocket and the hindgut wall of L3 larvae, namely *Achromobacter* (family Alcaligenaceae), *Bosea* (family Bradyrhizobiaceae), *Brevundimonas* (family Caulobacteraceae), *Citrobacter* (family Enterobacteriaceae) and *Pseudomonas* (family Pseudomonadaceae), qPCR with genus-specific primers was performed. In the pocket, *Achromobacter* was the most dominant of the genera, with an abundance about 10 times greater than that of *Pseudomonas* (**Figure 4**). *Citrobacter, Brevundimonas*, and *Bosea* showed lower abundances, with that of *Bosea* being three orders of magnitude lower than that of *Achromobacter*. The abundances of all four lower-abundant genera in the pockets differed significantly from that of *Achromobacter* (ANOVA, Tukey HSD test, $p < 0.05$). This is in line with the outcome of the 454-pyrosequencing, in which *Achromobacter* sp. (85% of family Alcaligenaceae sequences) was the most dominant of the identified genera in the pocket (**Figure 3**). However, since it was not possible to classify the family Micrococcaceae at the genus level, it must be taken into account that *Achromobacter* sp. may be overcome by a Micrococcaceae-related genus.

In the hindgut wall, the abundances of *Pseudomonas, Brevundimonas*, and *Bosea* spp. (*Pseudomonas* > *Brevundimonas* > *Bosea*) were in good agreement with their respective family abundances showed by the 454 pyrosequencing approach. The occurrences of *Citrobacter* and *Achromobacter* spp., respectively, the first and second most ubiquitous genera according to the qPCR outcome, matched their respective abundances in the L3 hindgut wall pyrosequencing (families Enterobacteriaceae and Alcaligenaceae, respectively), but were significantly higher than their abundances in L2 hindgut wall pyrosequencing (**Figure 3**). This outcome fits with the abovementioned idea that the relative abundances of the gut bacterial community members are dynamic depending on the larval instar.

PHB Detection in Pocket Isolates and Pocket Tissue by Nile Blue Staining and GC-MS

Considering the relatively close phylogenetic relationship between the major genus in *M. hippocastani* pockets, *Achromobacter* sp., and the PHB-accumulating bacterium that colonizes the *R. pedestris* midguts crypts, *Burkholderia* sp. (Kim et al., 2013), we speculated that PHB accumulation could also take place in the pocket symbionts. To test this hypothesis, pocket symbionts were isolated in selective media. The bacterial species that were retrieved are listed in **Table 1**. PHB accumulation was suggested in *Achromobacter marplatensis*, *Stenotrophomonas maltophilia*, and *Phyllobacterium myrsinacearum* by its positive fluorescence under UV light when cultured in Nile Blue agar (**Table 1**) (Ostle and Holt, 1982).

Gas chromatography coupled with mass spectrometry (GC-MS) of pocket tissue as well as isolated *A. marplatensis* was conducted in order to confirm PHB presence. For the analysis, pockets and bacterial mass were derivatized through *trans*-esterification with methanol in the presence of acid (see Materials and Methods) prior to injection into the gas chromatograph. The resulting chromatograms (**Figure 5**) showed a peak corresponding to 3-hydroxybutyric acid methyl ester, the derivatized 3-hydroxybutyric acid monomeric unit of PHB, with a retention time of 2.21 min (±0.01 min). Its identification was carried out by comparing the obtained mass spectrum and the retention time with the commercially available reference compound.

Raman Micro-Spectroscopy of the Pocket Tissue

In order to determine the spatial distribution of PHB-accumulating bacteria within the pocket pole, Raman micro-spectroscopy was performed. The Raman spectroscopic scans and spectra obtained are shown in **Figure 6**. Spectral unmixing using the N-FINDR algorithm revealed false-color images that showed different constituents by identifying different Raman spectral signatures. In the pocket poles containing

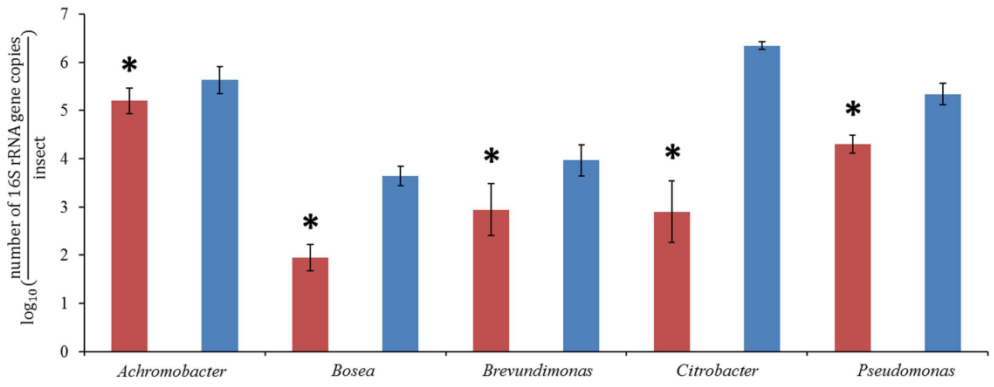

FIGURE 4 | quantitative real-time PCR (qPCR) of pocket samples (red) and hindgut wall samples (blue), obtained with genus-specific primers for *Achromobacter*, *Bosea*, *Brevundimonas*, *Citrobacter,* and *Pseudomonas* (ANOVA, Tukey HSD test, *$p < 0{,}05$, ±1 SD, $n = 10$).

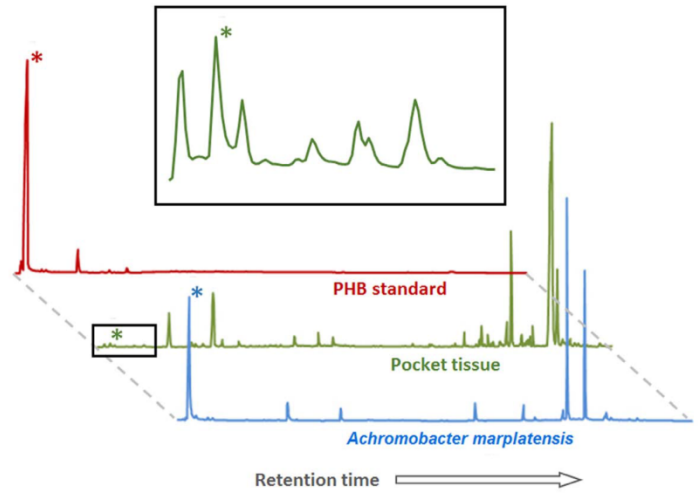

FIGURE 5 | Poly-β-hydroxybutyrate (PHB) detection by GC-MS in pocket tissue and *Achromobacter marplatensis* isolated from the pocket. The peak height reflects the relative abundance of the substance. Peaks marked with (*) correspond to the methyl ester of the 3-hydroxybutyric acid monomer of PHB. The area marked with a black square is enlarged above. The other peaks correspond to a variety of fatty acids of different chain lengths, common in both eukaryotic and prokaryotic cells, and to artifacts created by the method (peaks in the PHB standard chromatogram).

PHB-accumulating bacteria, they were distributed uniformly throughout the inner area of the pole as dots of approximately 1 μm diameter (spectrum 2 of **Figure 6B**, green area in false-color image). Within a typical bacterial Raman spectrum (Ciobotă et al., 2010; Majed and Gu, 2010), the presence of PHB granules was indicated by the bands at 837 and 1058 cm^{-1} (C-C stretching), and especially by the highly significant band at 1741 cm^{-1} (C=O stretching; compare PHB reference spectrum in **Figure 6C** with spectrum 2 in **Figure 6B**). The spectrum showing mainly C-C stretching (1067, 1131 cm^{-1}), CH_2 twisting (1299 cm^{-1}), and CH_2 bending (1444 cm^{-1}) vibrations (spectrum 1 in **Figure 6B**, blue area in false-color image), were likely derived from fatty acids, probably of a saturated nature as the bands that provide evidence of unsaturation were missing (1260, 1650, and 3023 cm^{-1}), whereas the bands that support saturation were strong (1299, 1444, CH stretch region at 2800 – 3000 cm^{-1}) (Wu et al., 2011). Finally, the spectrum of the mucus-like layer (**Figure 2C**) surrounding the inner part of the pole (spectrum 3 of **Figure 6B**, red area in false-color image) revealed a complex composition, consisting mainly of proteins with disulphide bridges (S-S stretch, band 500 and 505 cm^{-1}, respectively), high tyrosine (Tyr) content with bands at 650 cm^{-1} (C-C twist Tyr), 854 and 859 cm^{-1} (ring vibration Tyr), 1270 cm^{-1} (protein amide

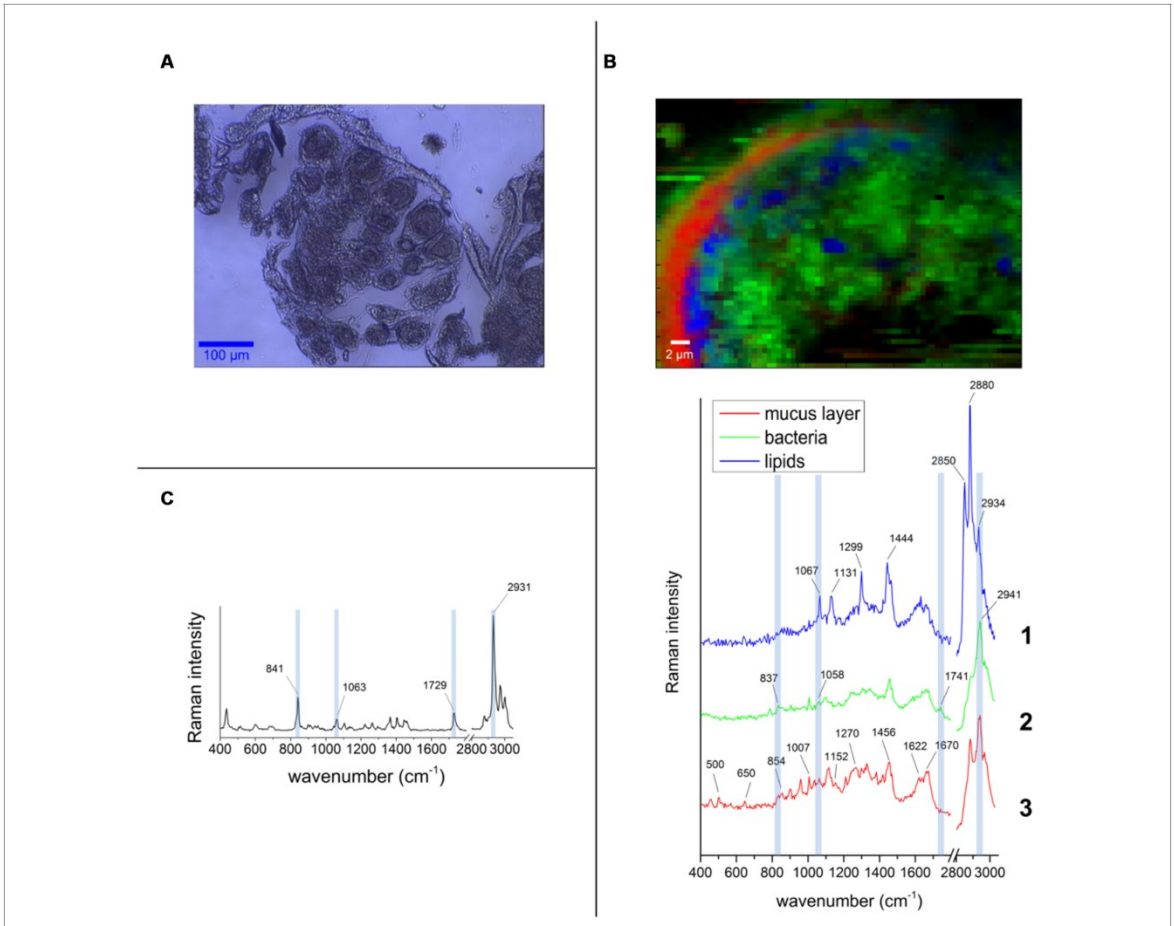

FIGURE 6 | False-color Raman images and spectra obtained from the pocket tissue with 532 nm excitation wavelength. (A) Bright field image of one of the tissue cuts used for the measurements. The round shape of the pocket poles is clearly visible. **(B)** Pocket pole with PHB-containing bacteria (green area in false-color image, spectrum 2). Spectrum 1 corresponds to fatty acids (blue area in false-color image) and spectrum 3 corresponds to the acellular mucus-like layer that surrounds the inner part of the pole epithelium (red area in false-color image). **(C)** Spectrum of the PHB pure substance is used as standard compound. The bands that are distinguishable in the spectrum from the pocket pole are highlighted in light blue.

III), 1456 cm^{-1} (CH_2 deformation), 1622 and 1626 cm^{-1}, respectively (C=C stretching Tyr and Trp), 1670 cm^{-1} (protein amide I or C=C stretching) (Tuma, 2005), and lipids (band 1270 cm^{-1} CH bend), and 1456 cm^{-1} (CH_2 deformation). For more detailed band assignment information, see Supplementary Table S4.

DISCUSSION

Bacterial Communities of the Hindgut Wall and the Pockets

Four hundred and fifty-four-pyrosequencing revealed that, in L2 larvae, the bacterial community of the hindgut wall was dominated by the families Pseudomonadaceae and Caulobacteraceae. These families, however, were overgrown in L3 by representatives of the family Porphyromonadaceae and the orders Bacteroidales and Clostridiales. Since these taxa are anaerobic, their proliferation in late larval instars may reflect a thickening of the bacterial layer attached to the hindgut wall, allowing the symbionts to reach more anaerobic areas toward the hindgut lumen, or a pronounced decrease in oxygen concentration due to high bacterial density. Similar shifts in bacterial abundances depending on the maturity of the larvae have been previously reported by Zheng et al. (2012) in *Holotrichia parallela* larvae. The hindgut wall of these larvae is populated by a reduced amount of coccoid cells in the L1 stage, although in the L3 stage, the density of bacteria is largely increased, with bacteroid cells dominating (Zheng et al., 2012).

In the adults, the relative abundances of Bacteroidetes, Proteobacteria (γ- and δ- classes) and the family Enterococcaceae (Firmicutes) were increased. Nevertheless, the overall composition of the adult hindgut wall community remained fairly constant compared to L3. This is in line with previous observations on *M. hippocastani* (Arias-Cordero et al., 2012). It was noted that the similarity between larval and adult bacterial communities becomes more evident in the later larval instars, suggesting that L3 larvae possess a community that is more closely related to that of the adults than to the L2 larvae. In addition, they noticed that the abundance of Enterobacteriaceae in the midgut increased continuously throughout the L2, L3, and adult stages (L2 < L3 < adult). In line with these findings is the presently observed increase of the genus *Citrobacter* from L2 to L3 (**Figures 3**, **4**). Such increase in abundance of *Citrobacter* representatives toward latter larval instars may be related to the increasing amount of ingested food as the larvae grow, as previously isolated *Citrobacter* sp. from the gut of *M. hippocastani* showed the ability to degrade xylan and starch in pure culture (Arias-Cordero et al., 2012). Furthermore, in adults, the high abundance of Enterococcaceae and Enterobacteriaceae representatives might be related to the shift to leaf-based diet, as these families showed resistance to tannins, an ubiquitous plant defense compound (Smith and Mackie, 2004; Singh et al., 2011).

The abundances of the bacterial genera in the L3 hindgut wall showed by qPCR (**Figure 4**) are in good agreement with the pyrosequencing result, being *Citrobacter* sp. dominant over *Achromobacter* sp., just as the Enterobacteriaceae family is more abundant than Alcaligenaceae in **Figure 3**. Contrary, *Achromobacter* sp. dominates in the pocket. This is also in line with the 454-pyrosequencing, where the sequences obtained clustered mainly within Actinobacteria and α- and β-Proteobacteria, taxa that showed very low abundances in the hindgut wall. This result highlights the singularity of the pocket bacterial community and suggest that they function as specialized symbiotic niches, analogously to previously described structures in other insects (Kikuchi et al., 2005; Grünwald et al., 2010).

Significance of the PHB Inclusions

Transmission electron microscopy unveiled a number of white cytoplasmatic inclusions in the pocket bacteria [potential poly-3-hydroxybutyrate (PHB)]. By GC-MS analyses, it was possible to confirm the presence of poly-3-hydroxybutyrate. Raman micro-spectroscopy revealed that PHB-accumulating bacteria are widely distributed throughout the lumen of the pocket pole. PHB is commonly accumulated by Eubacteria and Archaea and serves as a carbon reserve, stored in the form of water insoluble droplets in the cytoplasm (Rehm, 2003). Its presence is probably linked to the white cytoplasmatic inclusions observed in TEM. Likewise, PHB inclusions also are present in the endosymbiont *Burkholderia* sp. colonizing the midgut crypts of the bean bug *R. pedestris.* Each generation of this insect orally acquire the *Burkholderia* bacterium *de novo* from the environment, and the accumulation of PHB by the symbiont is crucial to ensure proper colonization of the crypts and correct development of the insect host (Kim et al., 2013). The colonization success by the PHB-accumulating symbiont could be related to its enhanced ability to cope with stress, as previous studies linked PHB accumulation to an increase of bacterial colonization efficiency and to tolerance to a variety of stresses such heat, reactive oxygen species, osmotic imbalance and nutritional depletion, among others (Kadouri et al., 2003; Kim et al., 2013). The high lipidic content within the pocket pole revealed by Raman micro-spectroscopy (**Figure 6**), suggests that the pockets are a nutritionally imbalanced habitat with a high C:N ratio that may favor the colonization by bacteria with the ability of accumulate PHB (Rehm, 2003). Also, oxygen limitation might contribute on selecting PHB-accumulating bacterial species over non-accumulating ones (Trainer and Charles, 2006). Symbiont sorting mechanisms in order to discard potentially pathogenic bacteria from the soil have been reported in the bean bug *R. pedestris* (Kim et al., 2013; Ohbayashi et al., 2015). However, in *M. hippocastani*, this putative discriminative process would not be as specific as in *R. pedestris*, since more than one bacterial phylotype are established in the pockets.

The presence of PHB is uncommon in vertically transmitted bacterial symbionts. Its accumulation is displayed mainly by free-living microorganisms, or by symbionts of environmental origin (Kim et al., 2013). This suggests that the PHB-accumulating pocket symbionts (*A. marplatensis* and possibly *S. maltophilia* and *P. myrsinacearum*; see **Table 1**) might be acquired from the environment. These genera, along with *Ochrobactrum thiophenivorans* (which is not likely to accumulate PHB; see **Table 1**), have been previously detected in the rhizosphere (Bertrand et al., 2000; Kämpfer et al., 2008; Ryan et al., 2009). Moreover, the BLAST alignments of the pocket isolates belonging to these taxa matched those of bacteria previously isolated from roots and soil (data not shown). Considering that, an environmental origin for these pocket symbionts is more plausible than a vertical transmission from mother to offspring. This latter possibility, nevertheless, cannot be totally discarded (Engel and Moran, 2013).

Physiological Role of the Pockets

The pockets in *M. hippocastani* have been only once described in literature (Wildbolz, 1954). Nonetheless, symbioses between insect and bacteria is a common and disparate phenomenon in nature (Douglas, 2009; Hansen and Moran, 2014) and analogous structures harboring symbiotic microorganisms have been found in other insects. Bugs belonging to the family Alydidae are associated with ectosymbiotic bacteria of the genus *Burkholderia.* It this case, the bacterium colonizes the crypts located in the distal section of the midgut (Kikuchi et al., 2005). Similarly, stinkbugs of the families Pentatomidae and Cydnidae harbor Gammaproteobacteria related bacteria in crypts located in the same region of the midgut (Prado and Almeida, 2009; Hosokawa et al., 2012). Other structures containing endosymbiotic bacteria and yeasts have been characterized in the proximal midgut of cerambycid beetles (Grünwald et al., 2010). The role of these symbionts within the insect gut and their involvement in host's nutrition, however, remains largely unknown.

In *M. hippocastani*, the pockets might be sites for denitrification processes. *A. marplatensis* isolated from these

small structures showed full denitrifying capabilities in a commercial nitrate reduction assay (**Table 1**). Moreover, the abundance of lipids within the pocket pole unveiled by Raman micro-spectroscopy (**Figure 6**) makes possible that these compounds are used by the pocket symbionts as electron donors for respiratory processes using nitrate as an electron acceptor (NO_3^-). Denitrification has already been reported in other rhizophagous white grubs (Majeed and Miambi, 2014). The presence of pockets could be also related to the rhizophagous diet of the larvae, as they were spotted in the rhizophagous larvae of *M. melolontha* as well (**Figure 1F**), but no similar structure was found in *Pachnoda marginata* (Supplementary Figure S4), whose grub-like larvae thrive not on roots but on humic acids. Host's diet and taxonomy have been pointed as key determinants of the composition of the gut symbiotic community by previous studies (Egert et al., 2003, 2005; Colman et al., 2012; Jones et al., 2013). Either way, it is possible that the pocket symbionts produce some kind of beneficial compound for the insect host. This hypothesis, however, remains for future research.

CONCLUSION

Our data revealed a complex and dynamic microbial community attached to the hindgut wall of the forest cockchafer. The composition of this community may be dependent on host's life stage. L3 larvae showed a more close community to the adults than L2 larvae. In addition, the presence of particular bacterial niches attached to the larval hindgut (pockets) is reported. Regarding the surrounding hindgut wall, these niches harbored a differentiated bacterial community in which the families Micrococcaceae and Alcaligenaceae were dominant. These structures could be related to denitrification processes. Furthermore, the presence of poly-β-hydroxybutyrate (PHB) granules among pocket bacteria is demonstrated. Further research is needed to fully understand the function of the pockets, and especially to determine the role(s) of the cytoplasmatic inclusions.

AUTHOR CONTRIBUTIONS

PA-P performed DNA extraction, light microscopy, pyrosequencing data analysis, gas chromatography measurements, isolation, identification and metabolic testing of symbiotic bacteria and prepared the samples for Raman analysis. Also wrote the manuscript. EA-C spotted the pockets in the gut. Performed light microscopy, DNA extraction, fluorescence *in situ* hybridization, pyrosequencing data analysis and TEM data analysis. Also contributed in writing the manuscript. AN designed and performed qPCR experiments and analyzed the data. Also contributed in writing the manuscript. CE performed the Raman micro-spectroscopy analysis and analyzed the data. Also contributed in writing the manuscript. JR contributed in the design of TEM experiments and in the analysis of the data. MK contributed in the analysis of pyrosequencing data and calculated richness indexes and rarefaction curves. Also contributed in writing the manuscript. MW prepared samples for TEM, performed analysis and contributed in the analysis of the data. Spotted PHB inclusions in TEM images. UN contributed in the analysis of the Raman data. Also contributed in writing the manuscript. WB had the main idea of the project and supervised it. Also contributed in writing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00291/full#supplementary-material

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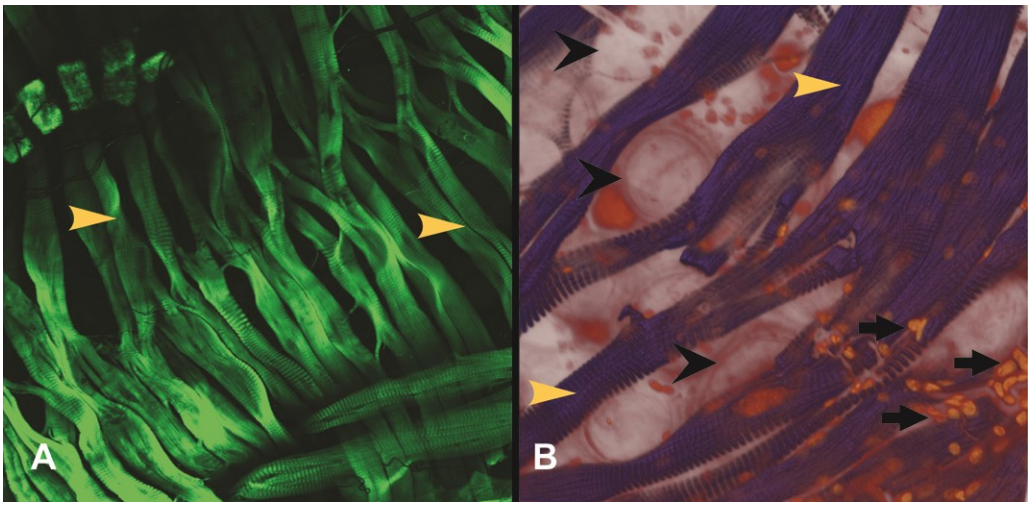
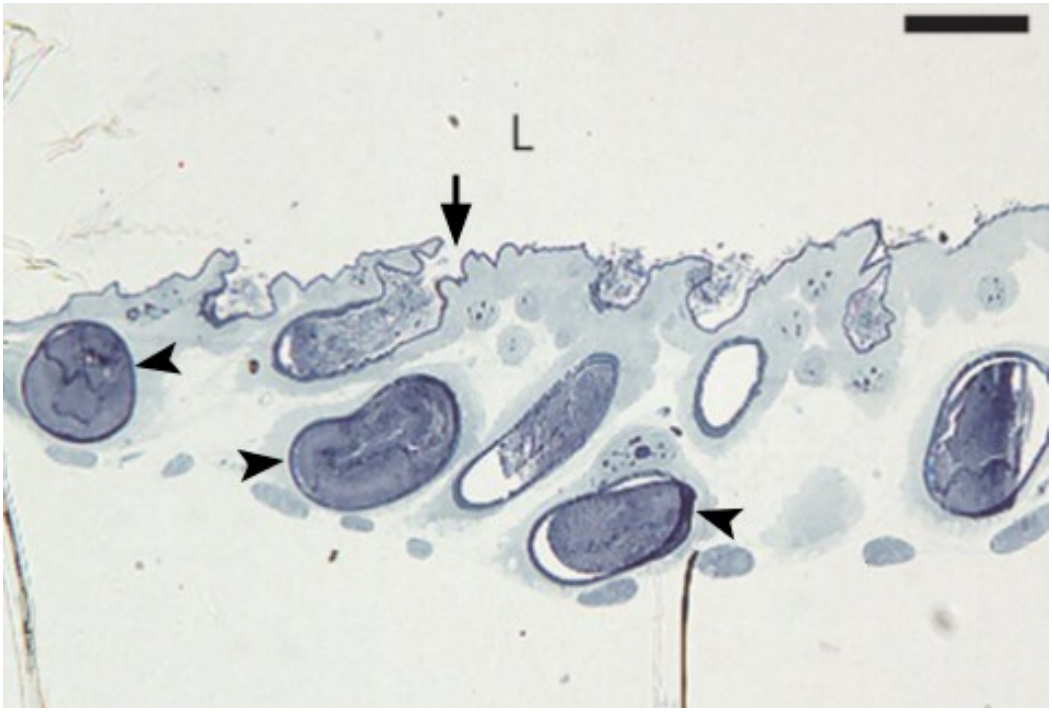
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

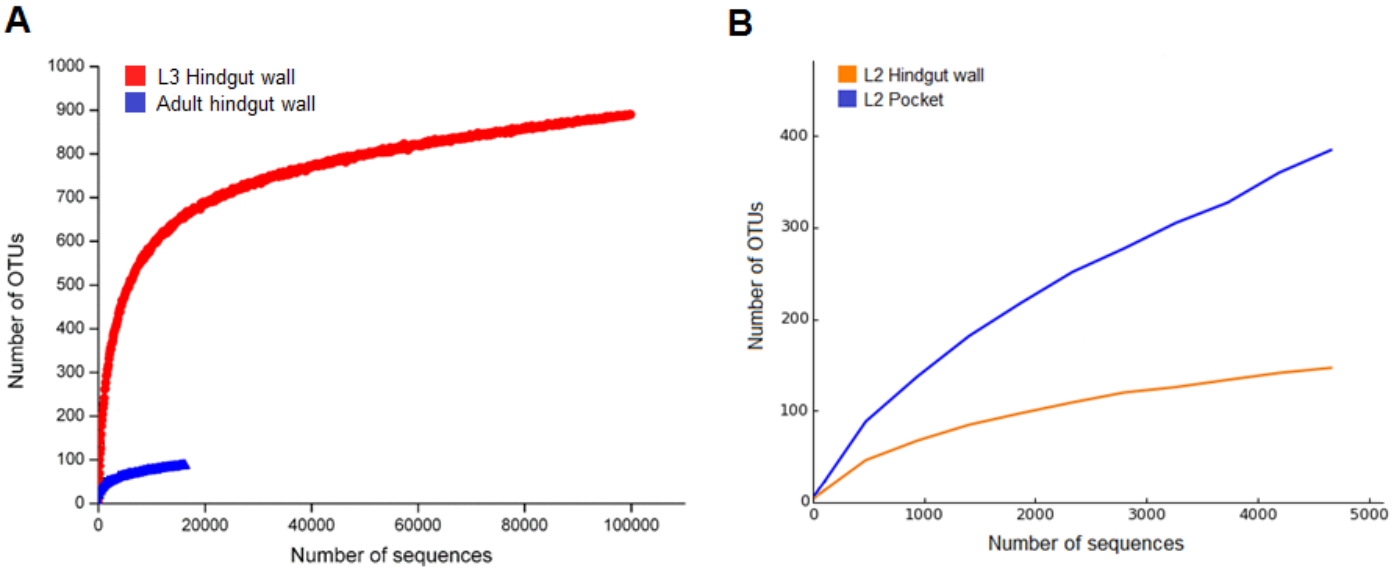
Supplementary Material



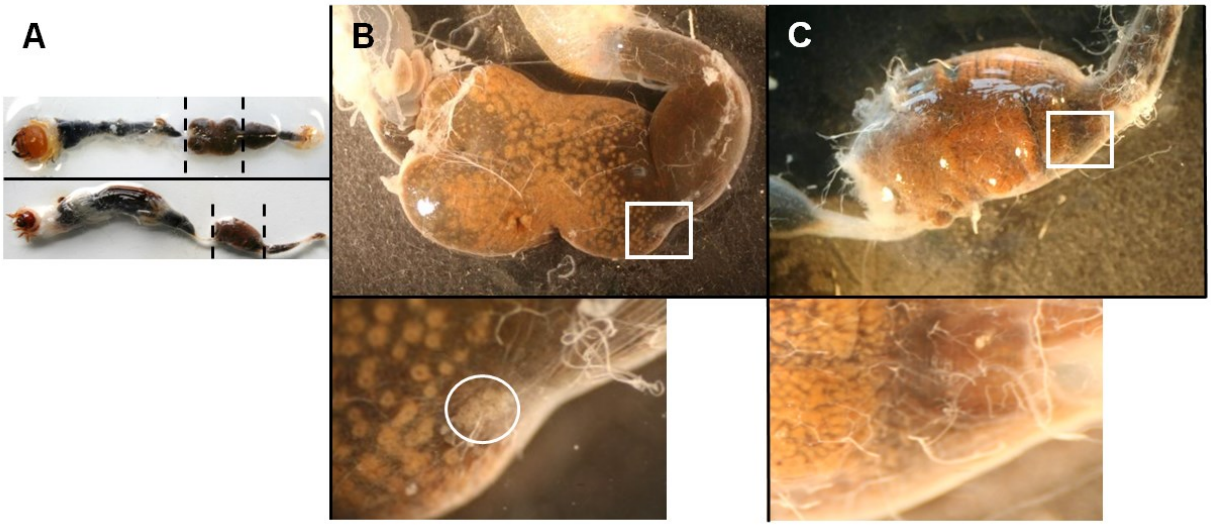
Supplementary Figure 1. Confocal images of the hindgut pocket tissue of a *Melolontha hippocastani* L2 larva. (A) Staining of the pocket tissue with Alexa Fluor 488 nm phalloidin stain (Phallotoxin, Invitrogen). (B) Double staining with Alexa Fluor 488 nm phalloidin stain and SYTOX Orange nucleic acid stain (Invitrogen), overlaid image. Yellow arrowheads point to muscle fibers that cover the pocket poles; black arrowheads indicate the position of the spheres at the distal point of the poles composing the pocket; black arrows point to the tracheoles that cover the pocket tissue.



Supplementary Figure 2. *Melolontha hippocastani* L2 larva hindgut and pocket microscopic detail. Cross section stained with Richards' solution. Black arrow point to the connection of one of the pocket poles to the hindgut lumen (L). Black arrowheads point to pocket poles. Scale bar 200 µm.



Supplementary Figure 3. Rarefaction curves of the 454-pyro sequencing. (A) Comparison of L3 hindgut wall and adults hindgut wall. (B) Comparison of L2 hindgut wall and L2 pocket.



Supplementary Figure 4. Absence of pockets in *P. marginata* larvae compared to in *M. hippocastani*. The area within the white square is enlarged beneath. (A) Overview of a whole larval gut of *M. hippocastani* (upper image) and *P. marginata* (lower image). The hindgut chamber is between the dashed lines. (B) Close-up of *M. hippocastani* hindgut chamber. The pocket is inside the white circle in the enlarged image. (C) Close-up of *P. marginata* hindgut chamber. Note the absence of pocket in the enlarged image.

Supplementary Table 1. Richness and diversity indices calculated at the OTU level from the pyrosequencing data of samples of pocket and hindgut wall of *Melolontha hippocastani*. Simpson expressed as 1-D, the bigger the number, the greater the diversity.

Sample	Total number of high quality reads	OTUs	Richness index	Diversity indexes	
			Chao1	Shannon	Simpson
Adults hindgut wall	16 016	74	105.67	3.06	0.71
L3 hindgut wall	85 233	572	705.91	6.52	0.96
L2 hindgut wall	4 797	147	217.38	2.87	0.67
L2 pocket	4 726	889	1338.04	4.19	0.78

Supplementary Table 2. Genus-specific primers used.

Primer	Target	Sequence (5'-3')	Reference
Achro F	*Achromobacter* spp.	GCTAATACCGCATACGCCCT	This study
Achro R	*Achromobacter* spp.	AGCCGTTACCCCACCAACTA	This study
Bos F	*Bosea* spp.	TAAGTTGGGAACTCTAGGGGG	This study
Bos R	*Bosea* spp.	TTTCGCTGCCCATTGTCACCG	This study
Brev F	*Brevundimonas* spp.	TTAGTTGGGAACTCTAATGG	This study
Brev R	*Brevundimonas* spp.	AGGATTAACCCTCTGTAGTTG	This study
Citro F	*Citrobacter* spp.	ACGGGTGAGTAATGTCTGGG	This study
Citro R	*Citrobacter* spp.	AGGTCCCCCTCTTTGGTCTT	This study
Pseudo F	*Pseudomonas* spp.	TTCGATTCAGCGGCGGACGG	This study
Pseudo R	*Pseudomonas* spp.	AGGTCCCCTGCTTTCTCCCGT	This study

Supplementary Table 3. Abundance of "Low abundance families" expressed as a percentage of total sample sequences. N.D.: not detected.

Family	Pocket L2	Hindgut wall L2	Hindgut wall L3	Hindgut wall Adult
Procabacteriaceae	0.0261	N.D.	0.1237	N.D.
Veillonellaceae	N.D.	N.D.	0.1396	N.D.
Proteobacteria phylum unk. fam.	0.1329	N.D.	N.D.	N.D.
Gammaproteobacteria class unk. fam.	N.D.	N.D.	0.1325	N.D.
Oxalobacteraceae	0.0057	0.0202	0.0721	0.0107
Microbacteriaceae	0.0403	N.D.	0.0545	N.D.
Nocardioidaceae	N.D.	0.0927	N.D.	N.D.
Bacteroidaceae	0.0591	0.0300	N.D.	N.D.
Opitutaceae	N.D.	N.D.	0.0708	N.D.
Chitinophagaceae	0.0641	N.D.	N.D.	N.D.
Methylobacteriaceae	0.0169	0.0395	N.D.	N.D.
Peptococcaceae	N.D.	N.D.	0.0514	N.D.
Turicibacteraceae	0.0438	N.D.	N.D.	N.D.
Bradyrhizobiaceae	0.0095	0.0202	N.D.	0.0027
Phyllobacteriaceae	0.0297	N.D.	N.D.	N.D.

Propionibacteriaceae	0.0019	0.0202	N.D.	N.D.
Moraxellaceae	0.0095	0.0102	N.D.	N.D.
Patulibacteraceae	0.0114	N.D.	N.D.	N.D.
Catabacteriaceae	0.0114	N.D.	N.D.	N.D.
Rhodobacteraceae	0.0114	N.D.	N.D.	N.D.
Staphylococcaceae	N.D.	0.0102	N.D.	N.D.
Coriobacteriaceae	0.0076	N.D.	N.D.	N.D.
Bacteroidia class unk. fam.	N.D.	N.D.	0.0005	0.0027

Supplementary Table 4. Raman bands assignment. Slashes (/) indicate different band positions with the same assignment. Hyphens (-) indicate interval. def.: deformation.

Observed band (cm^{-1})	Band assignment	References
500 / 505	S-S stretch	[121],[122]
650	C-C twist Tyr	[121],[122],[123]
837 / 841	C-C stretch	[124],[125]
854 / 859	Ring vibration Tyr	[121],[122]
1007	Phenylalanine	[121],[122],[123]
1058 / 1063 / 1067	C-O and C-C stretches	[124],[125],[126]
1114	C-C stretch	[126]
1131	C-O-H def., C-O and C-C stretches.	[126]
1152	C-N and C-C stretches	[123], [127]
1240 - 1280	$C-H_2$ twist, amide III	[126],[121],[122],[123],[127]
1440 / 1470	C-H deformation	[126],[121],[122]
1622 / 1626	C=C stretch Tyr and Trp	[122],[127]
1650 - 1680	amide I, C=C stretch	[121],[122],[127],
1729 / 1741	C=O stretch	[125],[124],[126]
2800 - 3000	$C-H_2$ and $C-H_3$ stretches	[124],[125],[126],[122]

4. Unpublished results

Differential gene expression analysis between *Melolontha hippocastani*'s pockets and the surrounding hindgut wall tissue

Pol Alonso-Pernas, Heiko Vogel, Wilhelm Boland

In preparation

In the unpublished results section, the outcome of a metatranscriptome comparison between hindgut wall and pockets of *M. hippocastani* raised in their natural habitat is showed. RNA was extracted using a commercial kit and sequenced in a Illumina HiSeq2500 platform. 250 bp paired-end reads were used to de novo assemble the transcriptomes. Contig annotation was carried out with BLASTx and Blast2GO PRO and differential expression analysis was performed in edgeR. Contigs aligned mainly to *Achromobacter* sp. in the pockets and to the Firmicutes phylum in hindgut wall. Host RNAs were expressed in the pockets in higher amounts than in hindgut wall. Gene expression suggest that pocket bacteria undergo aerobic metabolism and are exposed to higher levels of oxidative stress than the population of the hindgut wall. Hypothetical functions for the pocket might be immune-stimulation and regulation of host development, while the hindgut wall appears to be devoted to degradation of dietary polysaccharides and host nitrogenous wastes. Further research is necessary to experimentally prove these suggested roles.

1. Introduction

Insects are one of the most successful animals groups on earth and they thrive on a plethora of different diets. Although their digestive tracts present a wide variety of morphologies as result of adaptation to disparate food sources (Engel & Moran 2013) they are generally divided in the same three sections: the foregut, where the food is preprocessed and digestion may start; the midgut, where host digestive enzymes are secreted into the gut lumen and, in combination with symbiotic activity, most of the digestion and nutrient absorption takes place; and the hindgut, were water and other small molecules are absorbed (Chapman 2013). However, in some insect groups, this scheme can be subjected to significant modifications. For example, in termites or scarabaeid beetles, the hindgut is enlarged in the so called paunch (in termites) or fermentation chamber (in scarabaeids) and it also participates in digestion with the aid of symbiotic microorganisms (Calderon-Cortes et al. 2012). Or in *Spodoptera* larvae, the foregut is enlarged and accumulates α- and β-carotene, apparently as an adaptation to feeding on toxic plants (Shao et al. 2011).

In some cases, the morphological diversification of the digestive tract creates additional structures exclusive of particular insect taxa. These structures may be devoted to the housing of a specific bacterial ectosymbiont: for example, the midgut crypts in bugs of the Alydidae family are colonized by the environmental bacterium *Burkholderia* sp. (Kikuchi 2005) or by Gammaproteobacteria in bugs of the Pentatomidae and Cydnidae families (Prado & Almeida 2009; Hosokawa et al. 2012); also, the pouch-like cavity located in the midgut-hindgut junction of *Tetraponera* ants is occupied by root-nodule nitrogen fixing related bacteria (van Borm et al. 2002). In other insects, bacterial or fungal endosymbionts inhabit specialized cells (bacteriocytes or mycetocytes) which usually are grouped into clusters called bacteriome or mycetome. This is the case of the lygaeid *Kleidocerys resedae* or the chrysomelid *Bromius obscurus* that house gammaproteobacterial symbionts within, respectively, midgut or foregut-midgut junction associated bacteriomes (Küchler et al. 2010; Fukumori et al. 2017). Similarly, the cerambycid beetles *Tetropium castaneum*, *Rhagium inquisitor* and *Leptura rubra* harbour, respectively, unidentified yeast, *Candida rhagii* and *Candida shehatae* endosymbionts within mycetomes associated with the proximal midgut (Grünwald et al. 2009). The purpose of the microbes

inhabiting such symbiont-containing organs is unclear in most of the cases, although nitrogen fixing has been suggested for the root nodule-related bacteria within the paunch of *Tetraponera* ants (Stoll et al. 2007) and *Burkholderia* sp. associated with the bean bug *Riptortus pedestris* has been proven capable of degrading the insecticide fenitrothion (Kikuchi et al. 2012) and promoting insect growth and egg production through modulation of host protein expression (Lee et al. 2017). Regarding the endosymbiotic microorganisms confined within bacteriomes or mycetomes, it is commonly accepted that they provide a variety of essential nutrients to the host (Bright & Bulgheresi 2010).

RNA sequencing (RNAseq) has been successfully applied to explore the gut function of insects under changing conditions (Roy et al. 2016) as well as the interaction of insect host with either symbiotic (Peterson & Scharf 2016; Emery et al. 2017) or pathogenic organisms (Chen et al. 2017; Yadav et al. 2017). Tissue-specific RNAseq can also unmask differentially expressed genes and functions that could otherwise remain unidentified in whole-gut analysis (Mamidala et al. 2012; Shelomi 2017; Nakayama et al. 2017). In the present experiment, we used Illumina Hiseq to survey the transcriptome of the hindgut pockets of the forest cockchafer (*Melolontha hippocastani*). These symbiont-containing organs, placed at both sides of the terminal segment of the larval (male and female) hindgut chamber, were discovered in the 50s (Wildbolz 1954) and their architecture and bacterial community have been recently characterized in detail utilizing modern techniques (Alonso-Pernas et al. 2017). However, little is known about the processes ongoing in these enigmatic structures, such as the mechanism of symbiotic colonization or their role in the context of insect physiology. We expect to get an insight into these matters by comparing the pocket transcriptome with that of the surrounding hindgut wall of larvae retrieved straight from their natural environment.

2. Materials and methods

2.1. Insect collection and RNA extraction

Second-instar (L2) *Melolontha hippocastani* larvae were collected from a deciduous forest next to Pfungstadt (Germany, 49°49'44" N 8°36'17" E) in June 2016. Larvae were carried to the

laboratory in their natural soil and flash-frozen with liquid nitrogen immediately after arrival in order to minimize changes in gene expression. Frozen larvae were stored at -80 °C until further processing. Diethyl pyrocarbonate (DEPC, Sigma Aldrich, Saint Louis, MO, USA) treated phosphate buffered saline solution (PBS) and ddH_2O (Nagy et al. 2007) were prepared by adding 0.1% v/v DEPC into PBS (composition per liter: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 (pH 7.4)) or ddH_2O and incubating the mixtures at 37 °C for 24h before autoclaving. Dissection of thawed insects was carried out on ice in DEPC-treated PBS using ethanol sterilized tools rinsed with DEPC treated ddH_2O. Entire guts were carefully pulled out from the larvae. Pockets and 1x1 mm pieces of nearby hindgut wall were excised from the hindgut chamber and rinsed in DEPC-ddH_2O to remove debris and unattached bacteria. Tissues were pooled together in a 2 mL polypropylene screw cap micro tube (Sarstedt, Nümbrecht, Germany) forming 3 hindgut wall replicates, each consisting of 8 hindgut wall fragments from 8 different individuals, and 1 pocket replicate, consisting of approximately 100 pockets of 50 different individuals. Reduced size of pocket tissue prevented the obtainment of more replicates. Tubes containing dissected tissues were kept in liquid nitrogen until pooling was completed. RNA extraction was performed using the innuPREP RNA Mini Kit (Analytik Jena, Jena, Germany) with an extra homogenization step at the beginning of the protocol: 450 µl of Lysis Solution RL, 6 µL of lysozyme solution (50 mg/mL) and bashing beads were added to the sample 2 mL PP micro tube before homogenization with a 2010 Geno/Grinder® device (Spex®SamplePrep, Metuchen, NJ, USA) during 1 min at 1250 rpm. Samples were incubated for 3 min at RT and then homogenized again under abovementioned conditions. Rest of RNA extraction was conducted according to manufacturer's instructions. RNA quality and purity was tested with a Experion™ RNA chip (Bio-Rad, Hercules, CA, USA) on a 2100 Bioanalyzer (Agilent, Santa Clara, USA) following manufacturer's protocols.

2.2. RNA sequencing and de novo transcriptome assembly

RNA samples were sent on dry ice to the Max Planck Genome Centre in Cologne (Germany) for sequencing on an Illumina HiSeq2500 platform. Prior to library preparation ribosomal RNA was depleted from the samples. Random primed cDNA libraries were prepared with a TruSeq RNA Sample Prep kit (Illumina, San Diego, CA, USA) and sequenced, yielding 8 million (4

gigabases, each hindgut wall library), and 12 million (6 gigabases, pocket library) of 250 bp paired-end reads. Library quality control, adaptor trimming and *de novo* transcriptome assembly were carried out using CLC Genomics Workbench (Qiagen, Redwood City, CA, USA). A reference transcriptome was generated by a combined assembly of both hindgut wall and pocket reads using CLC Genomics Workbench v10.1 software with standard settings and an additional CLC-based assembly with the following parameters: word size = 64, bubble size = 300; minimum contig length = 350 bp; nucleotide mismatch cost = 2; insertion = deletion costs = 2; length fraction = 0.7; similarity = 0.9. Conflicts among individual bases were resolved in all assemblies resolved by voting for the base with the highest frequency. Contigs shorter than 350 bp were removed from the final analysis. The two assemblies were compared according to quality criteria such as N50 contig size, total number of contigs and the number of sequence reads not included in the contig assembly. For each assembly, the 50 largest contigs were manually inspected for chimeric sequences. The presumed optimal consensus transcriptome was selected from the different assemblies based on the highest N50 contig size, lowest total number of contigs and lowest number of chimeric sequences in the 50 largest contigs. The parameters of contig assembly were the following: word size = automatic, bubble size = 300; minimum contig length = 350 bp; nucleotide mismatch cost = 2; insertion = deletion costs = 2; length fraction = 0.7; similarity = 0.9. The resulting final *de novo* reference assembly (backbone) contained 305,905 contigs (minimum contig size = 350 bp) with an N50 contig size of 1,037 bp and a maximum contig length of 32,815 bp. The raw sequencing data was deposited in the NCBI Sequence Read Archive under the accession numbers SRR6123467 (hindgut wall replicate 1 sequencing run 1), SRR6123468 (hindgut wall replicate 1 sequencing run 2), SRR6123465 (hindgut wall replicate 2), SRR6123466 (hindgut wall replicate 3) and SRR6123464 (pockets).

2.3. Annotation and differential expression analysis

BLAST searches were conducted on a local computer cluster against the NCBI nr database with BLASTx (e-value cut-off 1e-1) and saved as .xml files. Further transcriptome annotation was carried out with Blast2GO PRO (Conesa & Stefan 2008) using Gene Ontology terms and EC numbers. Digital gene expression analysis was carried out using CLC Genomics workbench v10.1 to generate BAM mapping files, and finally by counting the sequences to estimate

expression levels, using previously described parameters for read mapping and normalization. For read mapping, we used the following parameters: read assignment quality options required at least 60 % of the bases matching (the amount of mappable sequence as a criterion for inclusion) the reference with at least 94% identity (minimum similarity fraction, defining the degree of preciseness required) within each read to be assigned to a specific contig; mismatch cost = 2; insertion = deletion cost = 3; maximum number of hits for a read (reads matching a greater number of distinct places than this number are excluded) = 10; n-mer repeat settings were automatically determined and other settings were not changed. RPKMs (reads per kilo base per million mapped reads) (Mortazavi et al. 2008) were calculated from the raw read count to normalize the expression of each contig. Contigs with RPKM < 1 in all libraries were considered not transcriptionally active and were discarded from further analysis. Differential gene expression analysis between pocket and hindgut wall libraries was carried out using the R package edgeR (Robinson et al. 2010), using TMM (trimmed mean of M-values) normalized values of read CPM (counts per million) per contig (Oshlack & Wakefield 2009). Significance threshold of FDR (false discovery rate) corrected p value was set to 0.05. Since lack of replicates in one of the samples analyzed (pocket library) reduces statistical reliability in differential expression testing, the following components were included in the analysis: a) before TMM normalization, contigs with =< 1 CPM (counts per million) in all libraries were filtered out, regardless of their RPKM value. Therefore, genes with very low counts that may influence the accuracy of multiple testing were removed while maintaining tissue-specific expression patterns. b) Besides a FDR value =< 0.05, the requirement of fold change (FC) value > 10 or < -10 was imposed to define differentially expressed genes, thus discarding genes with low FDR value due to consistency among hindgut wall libraries but with little difference between hindgut wall and pockets. Additionally, in order to discriminate the major processes ongoing in pocket and hindgut wall, highly and differentially expressed (HDE) genes were selected by defining high expression as any RPKM 10 times above the library mean. Hindgut wall contigs were considered highly expressed when meeting such requirement in all three hindgut wall libraries. The web-based KEGG pathway mapping tool Search Pathway (http://www.genome.jp/kegg/tool/map_pathway1.html) was used to functionally annotate differentially expressed (DE) enzymes with associated EC code and to reconstruct metabolic pathways according to the Kyoto Encyclopedia of Genes and Genomes (KEGG).

3. Results

3.1. de novo transcriptome assembly and annotation

A total of four sequencing libraries (three for hindgut wall, one for pocket) were produced from larval *M. hippocastani* RNA. Each hindgut wall library contained, approximately, 8 million reads (4 gigabases). Each pocket library contained about 12 million reads (6 gigabases). Reads were assembled into a total of 305.905 contigs (Tables 1 and 2). After read alignment and RPKM normalization, contigs with very low expression (RPKM < 1 in all libraries) were discarded. The taxonomic assignment of contigs performed via Blast2GO PRO revealed that the most common eukaryotic alignment was the insect *Tribolium castaneum* (fam. Tenebrionidae) in both hindgut wall and pocket libraries, while the most common prokaryotic alignments were *Achromobacter* sp. (fam. Alcaligenaceae) in the pocket library and *Clostridium* sp. (fam. Clostridiaceae) in hindgut wall libraries (Fig. 1).

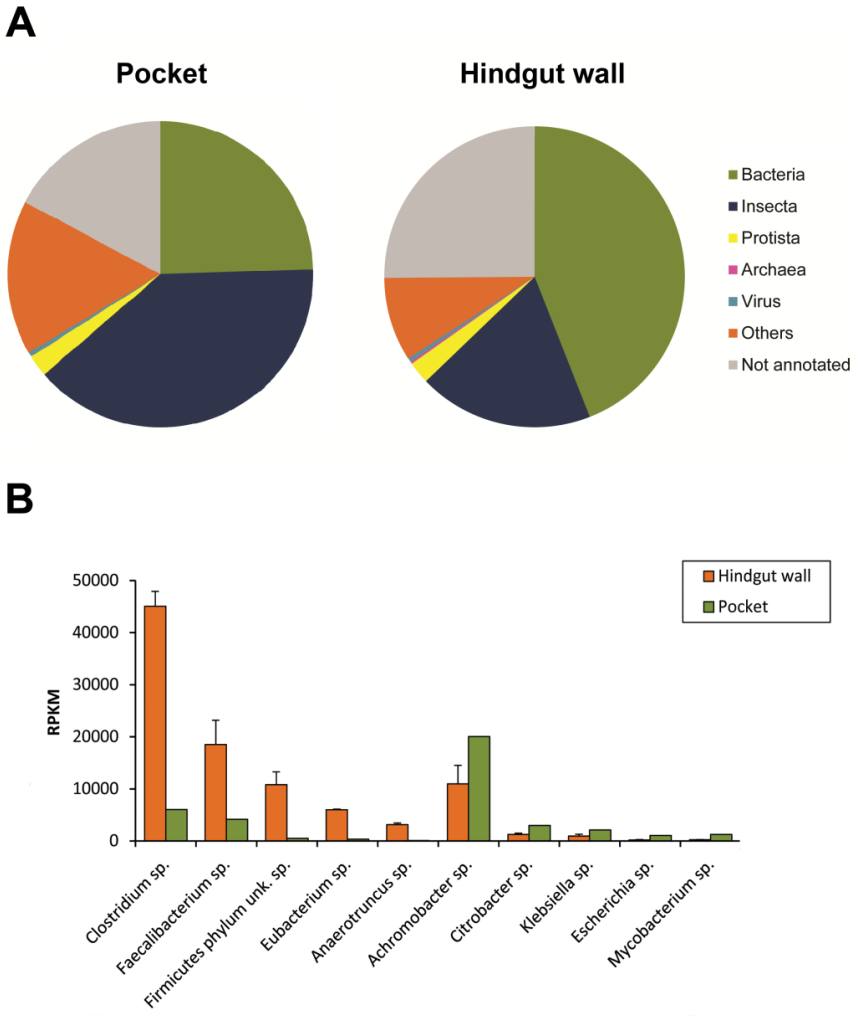


Figure 1 (previous page). Taxonomic distribution of annotated contigs. After RPKM normalization, contigs with RPKM < 1 in all libraries were discarded. A) Pie charts showing the distribution of contigs in taxonomic groups. Hindgut wall chart represents the mean of the 3 hindgut wall libraries. B) Bar chart showing the five bacterial genera with higher RPKM in hindgut wall libraries (left half) and pocket library (right half). Orange bars represent the mean of all three hindgut wall libraries. Error bars represent standard deviation.

Table 1. Contig length measurements

	With scaffolded regions	**Without scaffolded regions**
N75	636	631
N50	1,037	1,026
N25	1,984	1,948
Minimum	350	120
Maximum	32,815	32,815
Average	927	914
Count	305,905	309,982

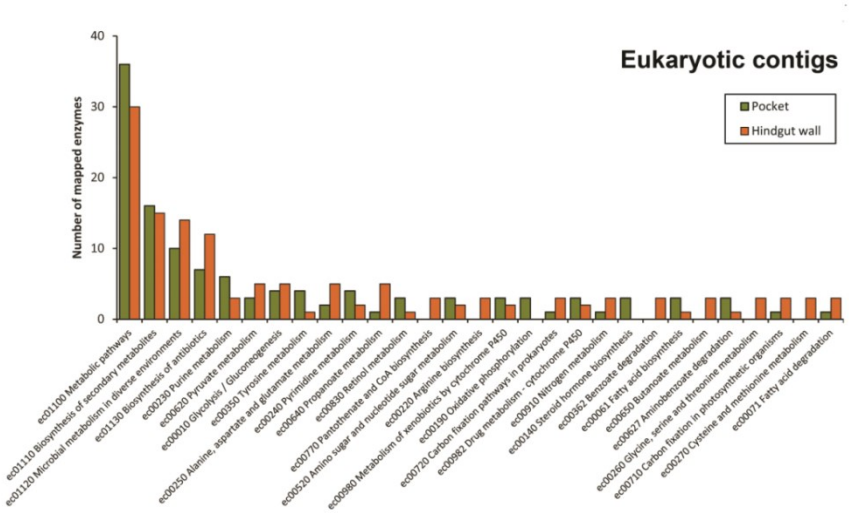
Table 2. Transcriptome assembly statistics

	Count	**Average length**	**Total bases**
Reads	64,067,071	227.73	14,590,016,540
Matched	51,171,136	227.1	11,620,962,785
Not matched	12,895,935	230.23	2,969,053,755
Reads in pairs	43,000,840	273.87	283,424,930
Broken paired reads	8,102,050	244.72	
Contigs	305,905	926	

3.2. Differential expression analysis

After filtering contigs with CPM =< 1 in all libraries, a total of 105,693 contigs were considered for differential expression analysis. Of these, edgeR identified 23,856 differentially expressed (DE) contigs (FDR<0.05, FC > 10 or < -10) between hindgut wall and pocket libraries. Differentially expressed KEGG pathways in pocket and hindgut wall were reconstructed by the web-based tool Search Pathway using as input contig-associated EC codes. A summary of the top DE KEGG pathways is shown in Fig. 2. A comprehensive list of all DE contigs and the KEGG pathways to which they map is shown in Supplementary Table 1. Additionally, any contig with RPKM ten times above the mean of the library was considered as highly expressed. By applying this rationale, 1,106 contigs were found to be highly expressed in all three hindgut libraries, while 2,657 contigs were found to be highly expressed in the pocket library. By combining these statistics, 495 contigs were found to be highly and differentially expressed (HDE) in the hindgut wall compared to pocket, and 220 were found to be HDE in the pocket compared to hindgut wall (Fig. 3). The top identified HDE contigs in pocket and hindgut libraries are shown in Table 3. A comprehensive list of all HDE contigs and the KEGG pathways to which they map is shown in Table S2, and can be summarized as follows: unique HDE genes in pocket library were, among others, 20 contigs that mapped to insect cuticular proteins and precursors, 15 to eukaryotic transposases and transposable elements, 9 to insect chitinases and chitin-binding proteins, 8 to bacterial porins, , 6 to eukaryotic reverse transcriptases, 6 to insect CD109 antigen, 4 to putative eukaryotic growth factors (3 of which are described as multiple epidermal growth factor-like domains), 4 to putative insect sulfate transmembrane transporters (sodium-independent sulfate anion transporter-like), 4 to eukaryotic histone-lysine N-methyltransferase and 3 histone-lysine N-methyltransferase-like, 3 to insect glucose dehydrogenases, 3 to insect Osiris proteins and precursors, 2 to toxic bacterial proteins of Hok family, 2 to insect yellow protein, 2 to proteins involved in insect response to stimulus (cuticular analogous to peritrophins 1-J isoform X2 and odorant binding 4), 2 to insect peroxidases and one to a bacterial peroxiredoxin, an insect serine protease H164 (EC:3.4.21), a bacterial lipoyl synthase (EC:2.8.1.8), an insect phenoloxidase subunit A3, a bacterial genetic competence-related protein, an insect arylphorin subunit alpha, a bacterial Tu translation elongation factor, an insect C-type lectin and an insect gram-negative bacteria binding protein. Unique HDE genes in

hindgut libraries were, among others, 10 contigs that mapped to bacterial flagellar proteins, 10 to bacterial carbohydrate ABC transporters and transcriptional regulators, 8 to bacterial proteases (6 of which are serine-type (EC:3.4.21; EC:3.4.17; EC:3.4.16)) and regulators, 7 to bacterial sporulation-related proteins, 6 to bacterial chemotaxis-related proteins, 5 to bacterial amino acid transmembrane transporters, 5 to bacterial proteins of pF06949 family, 4 to bacterial collagen repeats, 3 to bacterial aldehyde oxidoreductases (EC:1.2.99.7), 3 to bacterial aldehyde ferredoxin oxidoreductases (EC:1.2.7.5), 3 to insect cystathionine beta-synthases, 2 to bacterial carbamate kinases (EC:2.7.2.2), 2 to bacterial C4-dicarboxylate ABC transporters, 2 to bacterial iron transmembrane transporters, 2 to bacterial alkaline-shock proteins, 2 to bacterial beta lactamases, 2 to bacterial pyrophosphatases, 2 to bacterial addiction module toxin system, one to an insect aquaporin AQPcic, a bacterial ferredoxin, a bacterial manganese-containing catalase, a negative regulator of bacterial genetic competence, a bacterial citrate transporter and an insect histone deacetylase complex subunit SAP18.



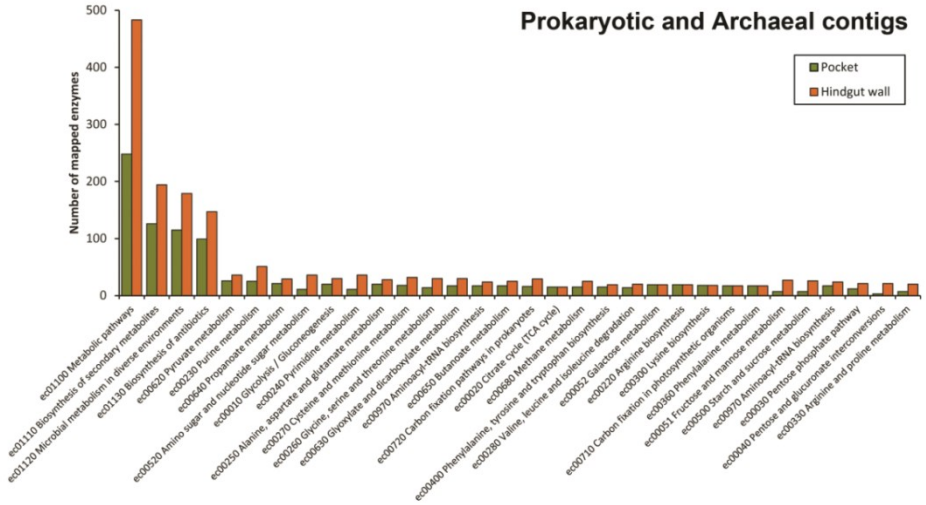


Figure 2 (previous page). Top DE KEGG pathways in pocket and hindgut wall tissues sorted according to contig taxonomic assignment.

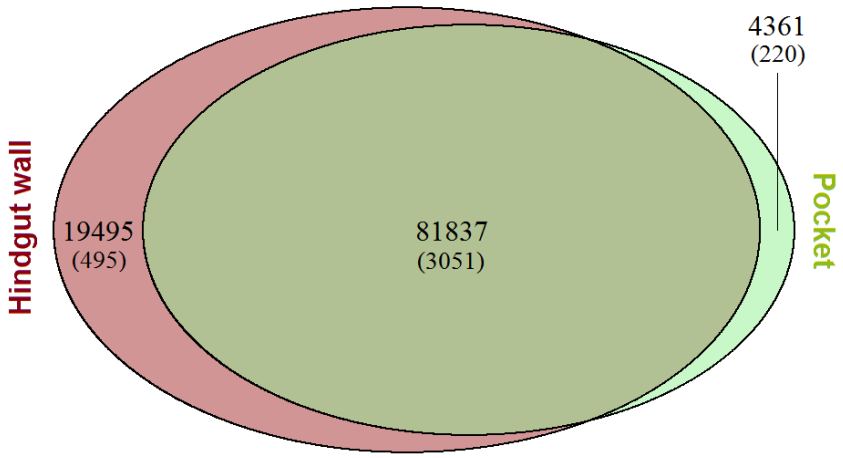


Figure 3. Venn diagram showing the distribution of contig expression between libraries. Brown area shows DE sequences in hindgut wall, green area shows DE sequences in pocket. Overlapping area shows sequences equally expressed in both tissues. Number of sequences (in parentheses, highly expressed sequences) contained in each area are given.

Table 3 (next page). Top identified HDE contigs in hindgut wall and pocket, sorted in decreasing order of fold change. Only contigs highly (RPKM > 10x library mean) and differentially expressed (FDR < 0.05, FC > 10 or < -10), annotated to proteins with known function are shown. In case of hindgut wall, $\log_2$ RPKM column shows mean of $\log_2$ transformed RPKMs of the three libraries. Fold change column shows the RPKM fold change compared to the other tissue.

HDE contigs in hindgut wall					
Contig name	**Description (BLAST2GO)**	**Organism**	**Log_2 RPKM**	**Fold change**	**Biological role**
Mhippo_Comb_C34879	alkaline-shock	*Pelotomaculum thermopropionicum*	5.78	26,035	Alkaline pH tolerance
Mhippo_Comb_C28060	phage infection	*Rattus norvegicus*	5.46	20,856	Membrane spanning protein, required for phage infection
Mhippo_Comb_C224	3D domain-containing	*Desulfosporosinus* sp.	5.26	18,162	Hydrolyzing O-glycosyl compounds, peptidoglycan turnover
Mhippo_Comb_C14542	tRNA delta(2)-isopentenylpyrophosphate transferase	*Carboxydothermus ferrireducens*	5.07	15,957	tRNA dimethylallyltransferase activity
Mhippo_Comb_C11085	transposase	*Pseudoalteromonas citrea*	4.93	14,424	Transposition, DNA integration
Mhippo_Comb_C5942	phenolic acid decarboxylase subunit C	*Blautia producta*	6.13	1,308	Aromatic compound catabolic process, response to toxic substance
Mhippo_Comb_C5739	flagellin domain	*Paenibacillus sabinae*	7.98	953	Bacterial-type flagellum filament
Mhippo_Comb_C6635	Cytochrome b561	*Tribolium castaneum*	5.40	480	Integral component of lysosomal membrane, transmembrane electron transport
Mhippo_Comb_C3434	Appr-1-p processing	*Dehalobacter* sp.	5.81	475	RNA-directed RNA polymerase activity, tRNA splicing
Mhippo_Comb_C7994	amino acid carrier	*Firmicutes bacterium*	5.76	448	Transmembrane transport, alanine:sodium symporter activity
Mhippo_Comb_C24847	alpha beta hydrolase	*Clostridium josui*	5.04	411	Hydrolytic activity (protease, lipase, peroxidase, esterase, epoxide hydrolase or dehalogenases)
Mhippo_Comb_C8677	uroporphyrinogen decarboxylase	*Treponema primitia*	5.44	408	Porphyrin-containing compound biosynthetic process
Mhippo_Comb_C4248	manganese containing catalase	*Anaerotruncus* sp.	5.67	406	Non-haem Mn-catalase, conversion of hydrogen peroxide to water and molecular oxygen
Mhippo_Comb_C23143	Na^+/H^+ antiporter	*Eubacterium* sp.	5.00	381	Transmembrane transport, antiporter activity

HDE contigs in hindgut wall (continued)					
Contig name	**Description (BLAST2GO)**	**Organism**	**Log_2 RPKM**	**Fold change**	**Biological role**
Mhippo_Comb_C2271	O-antigen polymerase	*Pelotomaculum thermopropionicum*	5.63	376	Lyase activity, lipopolysaccharide biosynthetic process
HDE contigs in pocket					
Mhippo_Comb_C263745	flexible cuticle 12	*Tribolium castaneum*	7.14	66,743	Structural constituent of cuticle
Mhippo_Comb_C276859	Hok Gef family	*Escherichia coli*	6.36	38,863	Bacterial toxin – antitoxin system
Mhippo_Comb_C205421	outer membrane porin	*Achromobacter piechaudii*	5.73	25,264	Porin activity, transmembrane transport
Mhippo_Comb_C28529	peptide of tmRNA	*Gordonia rhizosphera*	5.63	23,461	Ribosomal rescue system
Mhippo_Comb_C276989	IS116 IS110 IS902 family	*Rhodococcus erythropolis*	5.60	23,020	Transposase activity
Mhippo_Comb_C205423	outer membrane porin	*Achromobacter piechaudii*	5.59	22,813	Porin activity, transmembrane transport
Mhippo_Comb_C277394	RNA-directed DNA polymerase	*Escherichia coli*	5.44	20,614	RNA-dependent DNA biosynthetic process
Mhippo_Comb_C227062	pseudouridine-5 - monophosphatase	*Tribolium castaneum*	5.26	18,251	Hydrolase activity, structural RNA degradation
Mhippo_Comb_C276863	S-(hydroxymethyl)glutathione dehydrogenase	*Escherichia fergusonii*	5.13	16,618	Alcohol dehydrogenase (NAD) activity, formaldehyde detoxification
Mhippo_Comb_C205422	outer membrane porin	*Achromobacter arsenitoxydans*	4.98	14,978	Porin activity, transmembrane transport
Mhippo_Comb_C276858	TPA: cuticle	*Coptotermes formosanus*	7.95	14,179	Structural constituent of cuticle
Mhippo_Comb_C28536	peptide of tmRNA	*Mycobacterium kansasii*	7.39	13,616	Ribosomal rescue system
Mhippo_Comb_C101570	50S ribosomal L21	*Escherichia coli*	4.79	13,088	Cytosolic large ribosomal subunit protein
Mhippo_Comb_C270914	outer membrane porin	*Achromobacter piechaudii*	4.75	12,781	Porin activity, transmembrane transport
Mhippo_Comb_C199511	outer membrane A	*Achromobacter piechaudii*	6.89	12,760	Porin activity, ion transmembrane transport

4. Discussion

4.1. Taxonomic distribution of reads

As shown in Fig. 1A, the majority of annotated reads in pocket and hindgut wall fell into insect or bacterial taxa. Protist-annotated reads were also detectable in both tissues, although they are likely to originate from transient or parasitic microorganisms or from wrongly annotated insect-derived RNAs, as their symbiotic association with terrestrial coleopterans is exceptional (Tanahashi et al. 2017). Archaeal reads were also detected in both tissues, although in negligible amounts (less than 0.5%) which is in concordance with previous observations in *Melolontha melolontha* (Egert et al. 2005). Finally, around 10 and 20% of reads in hindgut wall and pocket tissue, respectively, mapped to non-insect eukaryotic organisms. Considering the lack of reference genome for *Melolontha hippocastani* and the inherent alignment uncertainty, all eukaryotic-mapped reads were assumed to be of host origin. Bacteria-mapped contigs in pocket and hindgut wall libraries fell into different genera depending on the tissue (Fig. 1B). In hindgut wall, reads mapped mostly to anaerobic gram-positive bacteria, while in the pocket they mapped mostly to aerobic or facultative anaerobic gram-negative bacteria. Differences in community composition between pockets and hindgut wall were previously observed; however, the Clostridiales and the Clostridiaceae, the order and family with highest number of mapped reads in the hindgut wall, were only poorly detected in the hindgut wall of second-instar larvae (Alonso-Pernas et al. 2017). They were, nevertheless, very abundant in third-instar larvae. This suggests that, although the larvae used in the present study had the body size corresponding to second-instar larvae, the composition of their gut microbiome was already drifting to third-instar's. Following this hypothesis, negligible number of reads mapped to the Micrococcaceae family in pocket libraries, although they were very abundant in second-instar larvae (Alonso-Pernas et al. 2017). This suggests that this bacterial family is either dormant or ultimately outgrown by the Alcaligenaceae family, concretely by the genus *Achromobacter*.

4.2. Highly and differentially expressed (HDE) genes in hindgut wall

The disparate annotation of highly and differentially expressed (HDE) contigs in pockets and hindgut wall is indicative of functional differences between the two tissues. In hindgut wall, a bacterial alkaline shock gene showed the highest fold change compared to pockets, which is in line with the alkaline pH (around 8) of this section of the gut (Egert et al. 2005) (Table 3). Also, many bacterial genes related to sporulation were HDE in hindgut wall (Table S2), which is not surprising due to the high abundance of Clostridial RNAs in the libraries. Sporulation-related genes are commonly expressed in gram-positive symbionts of insects and other organisms (Margulis et al. 1998; Paredes-Sabja et al. 2011) as a response to biofilm formation and high bacterial density (Dapa et al. 2013; McKenney et al. 2013). Other HDE genes suggesting ongoing bacterial colonization of the hindgut wall and microbe-microbe interactions were flagellar and chemotaxis-related contigs (Rawls et al. 2007; Wang & Wood 2011; Stephens et al. 2015), the toxin-antioxin system addiction module (Wang & Wood 2011), bacterial collagen repeats that might anchor bacteria in the gut wall and participate in host-microbe interactions (Yu et al. 2014) and beta-lactamases that may protect bacteria from antibiotics secreted by neighbouring microorganisms (Chen et al. 2017) or from toxic dietary compounds (Allen et al. 2009). All these HDE genes related to symbiotic colonization and interactions indicate that the hindgut wall community of *M. hippocastani* is not stable but subjected to continuous changes, which agrees with previous observations (Alonso-Pernas et al. 2017). HDE bacterial aldehyde oxidoreductases might neutralize antimicrobial agents contained in the diet (Correia et al. 2016). HDE bacterial transcripts for iron transporters were possibily a consequence of limitation of this element, whose availability in the gut is tightly controlled by the insect (Nichol et al. 2002). Congruent with iron scarcity is the presence of a HDE bacterial manganese-containing catalase transcript, a non-heme catalase expressed under conditions of microaerophilic oxidative stress and iron depletion (Whittaker 2012). Many ABC transporters and transcriptional regulators involved in transmembrane transport of carbohydrates were also HDE by hindgut wall bacteria, reflecting their role, along with insect secreted enzymes, in the digestion of recalcitrant polysaccharides, which are degraded extracellularly and the resulting soluble saccharides are internalized for further processing (Martin 1983; Artzi et al. 2017). HDE bacterial protease transcripts (mostly serine proteases) and the alkaline pH occurring in the hindgut, which might

contribute to the solubilization of dietary proteins, supports the digestive role of the resident community (Vinokurov et al. 2006). HDE bacterial amino acid transmembrane transporters (most of which were identified as sodium:solute symporters) suggest occurrence of free amino acids, probably due to ongoing proteolysis or *de novo* synthesis from host nitrogenous waste (Alonso-Pernas et al. 2017). Differentially expressed (DE) urease (EC:3.5.1.5) and carbamate kinase (EC:2.7.2.2.) in hindgut wall support presence of ammonia in hindgut (Table S1), as the former produces it by hydrolyzing urea and the latter uses it as substrate. HDE insect cystathionine beta-synthases (EC:4.2.1.22), which plays a role in the synthesis of the essential amino acid cysteine, might be related to the production of cysteine-rich proteins that regulate symbiotic population (Futahashi et al. 2013). HDE insect aquaporin might be involved in water absorption from feces, a well-reported hindgut function (Campbell et al. 2008; Chapman 2013). Finally, HDE transcripts for insect histone deacetylase complex may suggest repression of host genes, which is in line with the low number of detected host RNAs (Fig. 1) (Martin & Zhang 2005; Haberland et al. 2009). Alternatively, insufficient sequencing depth might have caused masking of low-abundant insect RNAs by highly-abundant bacterial RNAs, in which case some of the host's functions may have gone unnoticed.

4.3. Highly and differentially expressed (HDE) genes in pocket

An obvious increase in host-related HDE transcripts was observed in the pocket library compared to hindgut wall, possibly due to histone-lysine N-methyltransferase mediated upregulation (Table S2) (Martin & Zhang 2005). Most of the host HDE transcripts in pocket were cuticle-related. Insect cuticle constitutes a defensive barrier in the external body as well as in foregut and hindgut (Chapman 2013). Among pocket HDE transcripts both insect structural constituents of cuticle and chitinases are found, which suggest simultaneous cuticle synthesis and degradation. This may indicate either cuticular renovation, as the old cuticle must be degraded before being replaced by the new one (Willis et al. 2012; Chapman 2013), or cuticle thickening as a response to external challenge (Li & Denlinger 2009; Bascuñán-García et al. 2010). The latter possibility is supported by early observations of the tissue (Wildbolz 1954) and by the presence of HDE eukaryotic epidermal growth factors. It has been shown that other functions of these proteins are protecting the integrity of the intestinal barrier, reducing colonization by

pathogens and attenuating the epithelial inflammatory response (Tang et al. 2016; Kim et al. 2016). Further hints pointing towards active host immune response in pocket tissue are the HDE insect transcripts for gram-negative bacteria binding protein, C-type lectin and HDE CD109 antigen-like proteins which might be involved in pathogen recognition and activation of host defenses (Kim et al. 2000; Zelensky & Gready 2005; Warr et al. 2008; Yazzie et al. 2015), for a phenoloxidase domain and a serine protease, both possibly involved in melanin synthesis (Ashida & Breyt 1995) and for a yellow protein, which positions the melanin pigment in the cuticle (Willis et al. 2012) among other defensive roles (Gretscher et al. 2016). Melanin synthesis in pockets is further supported by the fact that in some individuals the pockets appear stained black (Wildbolz 1954). Functions of this pigment are mostly defensive, i.e. wound healing or pathogen encapsulation (Nakhleh et al. 2017). This points towards a tight regulation of the pocket population by the innate immunity of the host which, in combination with the oxidative stress suggested by HDE insect peroxidases and bacterial peroxiredoxins (Lushchak 2010), may have the purpose of preventing hindgut anaerobic bacteria from colonizing the pockets. Additionally, host proteases may directly control symbiotic titer (Byeon et al. 2015) and yellow proteins appear to be up- and downregulated depending on seasonal conditions (Daniels et al. 2014; Vilcinskas & Vogel 2016) raising the question of whether seasonal changes have an influence in pocket gene expression. The high number of HDE contigs that mapped eukaryotic transposases, transposable elements and reverse transcriptases might be consequence of oxidative stress (Giorgi et al. 2011) or of host perception of pocket colonization as an infection event (Mhiri et al. 1997). In view of the high number of pocket reads assigned to *Achromobacter* sp. (Fig 1B), it is tempting to assume that host immune system is less detrimental to this genus than to the others. The mechanisms underlying this possible selectiveness are to be studied, but is it plausible that HDE insect C-type lectin is involved, since lectins can be used by certain bacteria to evade host anti-microbial peptides (Pang et al. 2016). Also, the presence of symbiotic *Achromobacter* in the pocket might stimulate host immune system as reported in other systems, thus conferring resistance against pathogens (Kim et al. 2016). HDE contigs mapping to porins were also very abundant in the pocket. These passive transporters are the most abundant proteins in the outer membrane of gram-negative bacteria and their high expression in pockets suggests an intense exchange of nutrients and ions between symbionts and environment (Galdiero et al. 2012). Interestingly, porin-assigned reads aligned mainly to *Achromobacter* sp., which supports

a significant role for this bacterium in the pocket. Porins are crucial for the symbiotic colonization of the squid light-emitting organ (Aeckersberg et al. 2001), for the infection of insect hemocoel by an entomopathogen and possibly for the colonization of the gut of its nematode vector as well (Van Der Hoeven & Forst 2009). Moreover, porins have been related to invasion of non-insect tissues and manipulation of host defenses by pathogenic bacteria (Provenzano & Klose 2000; Duperthuy et al. 2011). Collectively, these observations suggest that *Achromobacter* sp. is engaged in a porin-mediated colonization process that may contribute to the triggering of insect immune response. Unfortunately, considering the current data any speculation on the role of the pockets as a whole is still far-fetched. Nevertheless, previous reports (Alonso-Pernas et al. 2017) and the presence of HDE sulfate transmembrane transporters suggests that the pockets might participate in nutrient provisioning. It is also noteworthy the pocket HDE contigs mapping to the Osiris gene family. These proteins are exclusive of insects, and although their role is unclear, they show expression peaks at specific life stages (embryo, second instar larvae and pupae) and might be involved in insect development (Shah et al. 2012). Also the HDE insect arylphorin subunit alpha and glucose dehydrogenases point towards the pockets being involved in host development. Alrylphorins are ubiquitous proteins in *Melolontha* sp. and other insects that act as amino acid storage proteins. Their concentration in hemolymph increases during each larval stage to quickly drop at each molt, as their amino acid components are used for the synthesis of body tissues (Delobel et al. 1993; Chapman 2013). Expression of glucose dehydrogenases is highly correlated with that of 20-hydroxyecdysone, a major insect molting hormone, and is increased during metamorphosis (Cox-Foster et al. 1990). HDE arylphorins, Osiris family proteins and glucose dehydrogenases make tempting to speculate that the role of the pockets and, perhaps, their bacterial symbionts, might go beyond insect immunity and nutrition and embrace other aspects of physiology such as development, as it happens in the *Riptortus-Burkholderia* system (Lee et al. 2017) or *Aedes* mosquitoes (Coon et al. 2017).

4.4. Differentially expressed (DE) metabolic pathways in hindgut wall

Taking into consideration all differentially expressed (DE) enzymes (FDR<0.05, FC>10 or <-10) with associated EC codes, DE KEGG pathways in pocket and hindgut libraries were reconstructed. The taxonomic assignment of the BLAST top hit allowed separation in host- and

symbiont-derived pathways (Fig. 2, Table S1). Symbiotic DE enzymes belonging to the KEGG pathways purine and pyrimidine metabolism, and aminoacyl tRNA biosynthesis were DE in both pocket and hindgut wall libraries. This indicates ongoing DNA, RNA and protein synthesis, thus high symbiont growth and activity in both tissues. Within these pathways, allantoinases (EC 3.5.2.5), allophanate hydrolases (EC 3.5.1.54) and ureases (EC 3.5.1.5) were DE only in hindgut wall, suggesting degradation of insect nitrogenous waste, that is, uric acid and urea, to ammonia, as part of a nitrogen recycling mechanism (Alonso-Pernas et al. 2017). Proximity of the outlet of the Malpighian tubules, located in the midgut-hindgut junction, to the site of sampling of hindgut wall tissue supports this hypothesis, as Malpighian tubules collect hemolymph waste and pour it into the hindgut (Shelomi 2017). On the contrary, the glutamine synthetase pathway was DE by both pocket and hindgut wall symbionts, suggesting that pocket bacteria utilize hindgut-produced ammonia for the synthesis of amino acids. Symbiotic chitinases were DE only in hindgut wall, probably reflecting the usage of this cuticular polysaccharide by hindgut bacteria as nitrogen and carbon source, thus incidentally contributing in maintaining the optimal thickness of the intima layer for proper diffusion of nutrients (Indiragandhi et al. 2007). In the hindgut wall more DE symbiotic enzymes involved in KEGG pathways related to carbon metabolism compared to pockets were found (fructose and mannose metabolism and starch and sucrose metabolism). This is in line with the digestive role of hindgut bacteria discussed above. Cellulases were only DE by hindgut wall bacteria. Taken together, these observations indicate that pocket bacteria may not be directly involved in digestion of insect food. Symbiotic enzymes belonging to carbon fixation KEGG pathways such as the reductive TCA cycle and the Wood-Ljungdahl pathway were DE in hindgut wall. They may contribute to the supply of acetyl-CoA needed for bacterial metabolism, but also to the production of acetate which might be taken up by the insect. Bacterial acetogenesis is well documented within the digestive tract of wood-feeding termites and roaches (Warnecke et al. 2007; Zhang et al. 2010) as well as in other insects with disparate diets (Matson et al. 2011). High acetate concentration in the gut fluid of *Melolontha melolontha* and hydrogen accumulation in the midgut, but not in the hindgut, is in line with ongoing acetogenesis in the hindgut, as this process uses hydrogen as electron donor, thus preventing its accumulation (Egert et al. 2005). Hindgut wall symbionts might also provide to the host, nutrients such as niacin, pantothenic acid, biotin and pyridoxine, as suggested, respectively, by the DE KEGG pathways nicotinate and nicotinamide metabolism, pantothenate and CoA biosynthesis, biotin metabolism

and vitamin B6 metabolism (Gilmour 1961; Cohen 2015). Methanogenesis might happen in hindgut wall as well, as coenzyme-B sulfoethylthiotransferase (EC 2.8.4.1) is DE in libraries of this tissue. However, this is likely to be a minor process carried out by the small archaeal population (Fig. 1, Table S1) (Egert et al. 2005). Finally, the pathways yielding geranyl pyrophosphate, geranylgeranyl pyrophosphate and farnesyl pyrophosphate from pyruvate (within the KEGG pathway terpenoid backbone biosynthesis) were DE by hindgut wall symbionts, suggesting production of these precursors of monoterpenoids (geranyl-PP), diterpenoids and carotenoids (geranylgeranyl-PP) and sesquiterpenoids (farnesyl-PP). Terpenoids are commonly used by plants as defense against herbivores and many of them have antimicrobial properties (Mithöfer & Boland 2012). Furthermore, it has been shown that many bacteria are able to synthetize them (Yamada et al. 2014). However, to our knowledge there is no report on insect gut symbionts producing such compounds. A more plausible hypothesis is that geranylgeranyl-PP or farnesyl-PP might be taken up by the host and used as precursors for hormone synthesis. Geranylgeranyl-PP might be used to produce carotenoids as well. Carotenoids play a role in multiple physiological functions of the insect host (Heath et al. 2012; Sloan & Moran 2012). Host KEGG pathways having more DE enzymes in the hindgut wall, as compared to pocket were glycolysis/gluconeogenesis, pyruvate metabolism and pantothenate and CoA biosynthesis. Expression of these pathways, related to energy, CoA and acetyl-CoA production, is likely linked to the absorption of acetate and other short chain fatty acids through the hindgut epithelium (Bayon & Mathelin 1980; Terra & Ferreira 2009). Fatty acid biosynthesis KEGG pathways were DE as well, possibly related to high acetyl-CoA production rate in hindgut epithelial cells. Also KEGG pathways leading to synthesis of amino acids were DE in hindgut wall libraries (alanine, aspartate and glutamate metabolism, arginine biosynthesis and cysteine and methionine metabolism). This might be consequence of high availability of free ammonia and/or glutamate due to the degradation of insect waste nitrogen by hindgut wall bacteria (Alonso-Pernas et al. 2017). The KEGG pathway biosynthesis of antibiotics also showed higher number of DE mapped enzymes in hindgut wall, reflecting a possible expression of antimicrobial compounds in order to keep the symbiotic community under control (Garcia et al. 2010). However, the number of annotated contigs with assigned eukaryotic taxonomy is surprisingly low in hindgut wall compared to pockets (Table S1). This might be consequence of either low

host RNA synthesis or masking of host's transcripts by the highly abundant bacterial RNAs. In the latter case, it is possible that some host functions have been overlooked.

4.5. Differentially expressed (DE) metabolic pathways in pockets

In pocket libraries only a few KEGG pathways have more mapped enzymes of symbiotic origin compared to hindgut wall (Fig. 2). This might be a consequence of a lack of EC codes for many annotated enzymes, or might reflect that in second-instar larvae the pocket community is still ongoing a colonization phase and does not express yet all the particular functional pathways of the tissue. Two of the KEGG pathways DE by pocket symbionts were the TCA cycle and the synthetic pathway of protoheme groups from glutamate and Fe^{2+} (within porphyrin and chlorophyll metabolism). The expression of these pathways, together with HDE bacterial lipoyl synthase, supports occurrence aerobic metabolism and oxidative stress in pocket symbionts (Navari-Izzo et al. 2002; Girvan & Munro 2013). As mentioned before, no cellulases were DE in pocket, but enzymes processing oligo- and monosaccharides such as sucrose, fructose or maltose (within starch and sucrose metabolism) and glucose (within glycolysis/gluconeogenesis) were DE by pocket symbionts, suggesting that they might use the saccharides resulting from degradation of diet polysaccharides, such as cellulose and hemicellulose, in the hindgut. Pocket symbionts also DE more enzymes related to sulfur metabolism compared to hindgut wall. This, together with HDE insect sulfate transmembrane transporters in the pocket, suggest a role related to sulfur provisioning. Sulfur is needed for the synthesis of essential amino acids such as methionine and cysteine, the deficiency of which renders the insect vulnerable to plant defensive protease inhibitors (Broadway & Duffey 1986). In pockets, it appears that the insect differentially expresses all enzymes leading to eumelanin synthesis (within tyrosine metabolism pathway). This is in line with the host defensive deployment in this tissue already suggested by the analysis of HDE contigs (Nakhleh et al. 2017). An unexpected feature of the pockets is that the insect differentially expresses three enzymes of the KEGG pathway steroid hormone biosynthesis, one of which, the cholesterol monooxygenase (EC 1.14.15.6), is exclusive of the pathway. Steroid hormones may regulate the occurring pocket cuticular synthesis deduced from the HDE of insect cuticular proteins (Karlson 1989). Additionally, steroids might downregulate the synthesis of antimicrobial peptides, thus allowing symbiotic colonization (Gordya et al.

2016). Steroids are also involved in molting and metamorphosis (Niwa & Niwa 2016), supporting the abovementioned hypothesis of the pockets playing a role in host development. Insect chitin degrading and synthetizing enzymes were DE in pocket (within amino sugar and nucleotide sugar metabolism), in line with the pocket cuticular renovation or thickening previously discussed (Willis et al. 2012; Chapman 2013).

4.6. Conclusion

The present comparison of the gene expression of the pockets with the surrounding hindgut wall provided the first functional insight of these enigmatic structures. Combining the data of the present study with previous observations, we conclude that differences between the two tissues include, but may be not limited to: a) The active bacterial population in hindgut wall is mainly composed by anaerobes of the Firmicutes phylum, while those of the pockets is composed of aerobic and facultative anaerobes among which *Achromobacter* sp. is the most significant genus. b) The environmental origin of pocket symbionts is strongly supported by their taxonomy, their aerobic metabolism, their expression of transcripts related pathogen-like tissue colonization and the triggering of insect's innate immunity. c) The environmental conditions between the two tissues appear to be remarkably different, the pockets having a higher oxygen concentration and oxidative stress, and lower bacterial competition and concentration of dietary toxins than the hindgut wall. d) The hindgut wall is likely to be the site of bacterial degradation of dietary recalcitrant polysaccharides and host nitrogenous wastes, while the pockets might play a role in stimulating host immunity, regulating host development and/or micronutrient absorption. e) The pocket bacterial community probably varies across larval stages as does that of the hindgut wall; therefore, its gene expression may change depending on larval maturity. Time-course RNAseq experiments may be useful to clarify this question.

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Supplementary Material

The supplementary material of this manuscript can be downloaded at:

https://www.dropbox.com/s/mmqmcoedvidyaic/Supplementary%20materials%20RNAseq.zip?dl=0

5. General discussion

5.1 Summary of the findings

The purpose of this thesis was to deepen into the composition and function of the gut microbiome of two insects occupying two well-differentiated ecological niches, namely the coleopteran *M. hippocastani* and the lepidopteran *S. littoralis*, thus completing the work initiated by Arias-Cordero et al. and Shao et al. [114], [120]. In the case of *M. hippocastani*, the first step was to determine the relevant bacteria meriting further investigation among the complex symbiotic milieu suggested by Arias-Cordero et al. Illumina-SIP analysis allowed the identification of the bacterial families Lachnospiraceae and Enterococcaceae in larvae and Enterobacteriaceae in adults as significant cellulose degraders, and the genera *Burkholderia* in larvae and *Porphyromonas* and *Bacteroides* in adults as key players in the recycling of host nitrogenous waste (Article I). In *S. littoralis*, the bacterium *E. mundtii* was identified as a metabolically active symbiont across the whole larval stage and also in adult females [108], [120]. Thus, to get insight into its role, isolation of *E. mundtii* and genome sequencing were carried out, which revealed a complex machinery devoted to carbohydrate metabolism, suggesting an involvement of this symbiont in digestion of diet polysaccharides (Article II). Furthermore, this thesis aimed to gain an understanding of the dynamics of the bacterial community inhabiting the hindgut wall across various life stages of *M. hippocastani* (second- and third instar larvae and adults) and to survey the bacterial community colonizing specialized symbiotic niches (pockets) located at both sides of the distal section of the larval fermentation chamber. 454-pyrosequencing revealed that the composition of the bacterial community inhabiting the hindgut wall depends on host life stage, and that the bacterial population of the pockets is markedly different to that of the hindgut wall. This fact points towards a specific role for these small structures (Article III). Finally, in order to further understand the role of the pockets within the context of insect physiology, differential gene expression of second instar larvae's hindgut wall and pockets was performed. This confirmed that some genes were differentially expressed between the two tissues, being the hindgut wall possibly devoted to food digestion and nutrient provisioning and the pockets to host immunity and/or development regulation (Unpublished results).

5.2 Function of the gut bacterial symbionts of a coleopteran (*Melolontha hippocastani*)

As stated in the introduction, the bacterial community inhabiting the digestive tract of *M. hippocastani* is complex and relatively stable [16], [114]. Nine bacterial classes persist throughout the whole host life cycle despite the host radical change in ecological niche when it transits from larval to adult stages (underground root feeding larvae – aboveground leaf feeding adult). However, at lower taxonomic level variations in community composition are observed, presenting the adults with less bacterial phylotypes than the larvae [114]. These observations suggest: a) the significant symbionts for insect physiology shift taxonomically when entering the adult stage in order to adapt to host new diet and b) the taxonomic range of these symbionts is wider in larvae than in adults. The outcome of the first study presented in this thesis, involving Stable Isotope Probing coupled with Illumina sequencing (Illumina-SIP), is in line with both hypotheses (Article I). More bacterial families were detected and, of those, a higher number were isotopically labeled in larvae compared to adults, suggesting that larvae harbor a more complex bacterial network devoted to the processing of the experimental substrates used (cellulose and urea). This is especially noteworthy when looking at the bacterial families labeled with ^{13}C cellulose. In adults, exclusive and highly significant labeling of the Enterobacteriaceae family suggests that cellulose degradation might be uniquely carried out by these bacteria, although the participation in cellulose breakdown of extracellular enzymes secreted by other bacterial families which did not incorporate any labeling cannot be ruled out. In larvae, many bacterial families got enriched in labeled dense pooled fractions, as compared to control, but only Lachnospiraceae and Enterococcaceae tended to be significant. Although this result can be due to limitations of the technique, it is reasonable to speculate that the observed dilution of the labeling might be due to interspecific interactions and/or competition for the experimental substrate (crossfeeding) [128], [129]. In the context of the nutrient-poor root-based larval diet, which may contain up to 50% of cellulose and large proportion of lignocellulose and humic materials, cooperation of various bacterial taxa with complementary genetic capabilities would ensure complete digestion of the food and maximization of its nutritional yield. Examples of this synergy are well reported in humans, where Bifidobacteria degrade mucin released by Bacteroides [130] or in lower termites, where fermentation products released by protists are rapidly converted to acetate by bacteria [38]. *M. hippocastani*'s adult leaf-based diet is more nutritious than roots and less bacterial participation may be required to process it [131]. Considering the low nutritional value of the

larval diet, it is surprising that none of the cellulose-labeled bacterial families in larvae possess PICRUSt-predicted lignin degrading genes. Assuming that the predictions are correct, explanations of this fact might be either that the roots contain enough cellulose to fulfill the needs of the insect [132] or that lignin degradation is performed by other bacteria, such as Clostridiales [133]. The low relative abundance of putative lignin-degrading bacteria in *M. hippocastani* larval digestive tract, however, suggest that lignin degradation may be a minor process, if happening at all, and that the larvae may rely exclusively on cellulose and possibly hemicellulose to fulfill their carbon and energy needs.

By combining the Illumina-SIP results with previous studies it is also possible to draw inferences about the location of cellulose-utilizing symbionts within the gut. It was determined that in the larval midgut of *M. hippocastani* the most abundant microbes are facultative anaerobes belonging to the γ-proteobacteria class (Enterobacteriaceae and Pseudomonadaceae families). Little symbiotic colonization of the midgut epithelium and relatively high numbers of bacteria attached to the food material were also observed [114], suggesting that these bacteria might be transient, possibly ingested with the diet. This, together with the short residence time of the food in midgut (4 to 8 h in *M. melolontha*) compared to hindgut (up to 4 days) [115], suggest that little symbiotic cellulose digestion occurs in the midgut of *M. hippocastani* larvae. Host-secreted cellulases, however, may still act in this gut section and be dragged by the gut fluid to the hindgut, were they may further contribute to polysaccharide breakdown along with symbiotic bacteria [69], [134]. In contrast to the midgut, the hindgut epithelium is extensively colonized by Lactobacillales and Clostridiales [16], (Article III). Interestingly, the ^{13}C cellulose-labeled bacteria in larvae belong to these taxa, supporting the assumption that the larval midgut has no significant role in symbiotic digestion, although preliminary breakdown of polysaccharides by insect- or bacteria secreted enzymes may still occur. On the contrary, the family Enterobacteriaceae in adults was clearly labeled by ^{13}C cellulose, which might suggest an increase of the cellulolytic activity in adult midgut compared to larvae and/or that a rise in oxygen concentration in the smaller adult hindgut acted detrimentally towards the predecessor anaerobic community, allowing the facultative anaerobic Enterobacteriaceae family to dominate.

A clear enrichment of *Burkholderia* sp. in labeled dense pooled fractions of larval ^{15}N urea gradient suggests involvement of this genus in the recycling of nitrogenous waste. Isotope Ratio

Mass Spectrometry (IRMS) of various insect tissues demonstrated incorporation of urea-derived ^{15}N in the insect body, achieving the larvae higher δ values than the adults. Both results are congruent with the lower nitrogen content of roots compared to leaves [135], which demands an efficient nitrogen recycling mechanism. Furthermore, IRMS also revealed differences in ^{15}N incorporation between tissues, of which the most striking one is between adult fat body and adult muscular tissue. Insect fat bodies have known implication in storing urates [136] and fixing ammonia back into amino acids [137], sometimes with the aid of obligate symbionts [138]. Thus, IRMS results might indicate that in *M. hippocastani* the fat bodies participate in the treatment of nitrogenous waste, serving at least as storage sites. To determine whether *M. hippocastani* harbor microbial symbionts in the fat bodies requires further investigation; in case they exist, those putative fat body-associated nitrogen recycling bacteria may be taxonomically different to those of the gut. To my knowledge, none of the ^{15}N labeled bacterial genera in adults (*Parabacteroides* and *Bacteroides*) is reported to migrate across insect organs. This might not be the case in larvae, as a *Burkholderia*-related obligate symbiont colonizes the fat bodies of the scale insect *Acanthococcus aceris* and migrates to the ovaries during oocyte development to ensure vertical transmission [139]. Interestingly, as suggested by the PICRUSt predictions, more bacterial taxa might be involved in the recycling of nitrogen, as ureolytic enzymes were not predicted to be present among labeled bacteria. Those unknown urea degraders might be other gut bacterial families such as Micrococcaceae or Clostridiaceae (colonizing the second and third-instar hindgut wall (Article III)) [140],[141], which might release ammonia to the lumen and without incorporating detectable levels of ^{15}N in their DNA.

5.3 Function of *Enterococcus mundtii* within the gut of a lepidopteran (*Spodoptera littoralis*)

Based on the analysis of the genome of *E. mundtii*, the dominant gut symbiont of *S. littoralis*, inferences about the potential roles of this bacterium within the gut can be made (Article II). As already mentioned in the article, the most striking feature of symbiotic *E. mundtii* is the large amount (almost 12%) of genome coding capacity dedicated to carbohydrate transport and metabolism genes, including ABC-type sugar transporters, sugar binding proteins and multiple glycosyl hydrolases. This is not surprising due to the carbohydrate degrading capabilities that the strain showed *in vitro* and considering its ecological niche, the herbivore gut, which contains abundant plant-derived saccharides [120]. Furthermore, the predicted pathways suggest that

symbiotic *E. mundtii* has the genetic tools for fermentative production of short chain fatty acids such as formate or acetate. The generation of these compounds may provide two benefits: first, symbiont-produced short chain fatty acids, specially acetate, may be taken up by the insect host and used as carbon and/or energy source [87], [142]; second, since these are acidic compounds, they might contribute in lowering the cytoplasmic pH, thus protecting the symbiont against the harsh alkalinity of its main habitat, the lepidopteran midgut [34],[119],[143]. Other genes found in symbiotic *E. mundtii* potentially involved in balancing the intracellular pH are several Na^+/H^+ antiporters and amino acid deaminases, as well as cardiolipin synthases. Their synthesis product, the membrane lipid cardiolipin, is increased upon alkaline challenge in *Bacillus* sp. and might function as a proton trap [143]. Other crucial traits for the continuity of *E. mundtii* in the digestive tract that are reflected in its genome are the production of the antimicrobial peptide mundticin, a bacteriocin that kills invading bacteria by forming membrane pores [144]. Also, polysaccharide biosynthesis genes [145], toxin-antitoxin systems (addiction module, PIN family, MazF and HicA, among others) [146], D-alanyl-lipoteichoic acid biosynthesis genes and teichoic acid ABC transporters [147] might play a role in the formation of the observed intestinal *E. mundtii* biofilm [34],[120]. D-alanyl esters of enterococcal lipoteichoic acid are reported to act against antimicrobial peptides as well [147]. This brings up the possibility of this molecules protecting *E. mundtii* against harmful peptides secreted by competing bacteria. Protease-encoding genes were widely represented in the genome of symbiotic *E. mundtii* (metalloproteases, cell wall associated protease, serine protease, carboxypeptidases, among others). In an environment rich in plant defensive protease inhibitors like the lepidopteran gut, the bacterial overproduction of inhibitor-sensitive proteases, the expression of inhibitor-insensitive protease isoforms or the activation of proteases that hydrolyze protease inhibitors might counteract the inhibitory effect [148]. Further support of a role for *E. mundtii* in detoxification are the presence of genes encoding for cytochrome P450 and carboxylesterase which might provide pesticide resistance to the host [148],[149]. Furthermore, the presence of glycosyl transferases might reduce toxicity of plant phenolic compounds by conjugation with sugars [148] and phosphodiesterases might be involved in pesticide and xenobiotic degradation [150]. In summary, the gene repertoire of symbiotic *E. mundtii* suggest that this bacterium has the ability to provide several benefits to its host *S. littoralis*, perhaps being the most significant one the degradation of diet polysaccharides.

5.4 Dynamics of the bacterial community inhabiting the hindgut wall of larval *Melolontha hippocastani*

Although a core microbial community remains constant during the entire life cycle of *M. hippocastani* (see Introduction), local taxonomic shifts depending on the stage of host development are observed. Previous work determined that in the larval midgut and adult gut the family Enterobacteriaceae presents the larger number of Operational Taxonomic Units (OTUs). However, other symbiotic families were detected only in certain developmental time points (i.e. the family Chitinophagaceae was detected only in second instar larvae and adults, the family Acidaminococcaceae was detected only in third instar larvae and adults) [114]. 454 pyrosequencing of hindgut wall tissue of second- and third instar larvae and adults determined that bacterial taxonomic shifts occur in the hindgut wall as well (Article III). Summarizing, the bacterial families Pseudomonadaceae and Caulobacteraceae were dominant in second instar larvae, while in third instar the most abundant bacterial phylotypes were the Bacteroidales and Clostridiales orders. It was between these two larval instars, second and third, where the biggest variation in community composition occurred. The adult bacterial community was fairly similar to third instar larvae, with increased abundances of the Bacteroidales order and the Enterobacteriaceae, Desulfovibrionaceae and Enterococcaceae families, and a decreased abundance of the Clostridiales order. These observations suggest a role of oxidative stress in shaping the symbiotic community: in second instar larvae, when the hindgut chamber is smaller and therefore more aerobic, the symbiotic milieu is dominated by families performing aerobic respiration [151]. The opposite scenario happens in third instar larvae, escalated by the fact that the biofilm lining the hindgut wall is likely to be thicker and thus the bacteria are able to reach more anaerobic zones of the lumen. Consequently, anaerobic bacteria dominate the hindgut wall symbiotic community of third instar larvae [151].

Nevertheless, the regulatory apparatus of such symbiotic shift most likely goes beyond the mere presence or absence of oxygen. A plethora of host-, microbe- and host-microbe interplay derived mechanisms has been reported to trigger multiple stresses that keep symbiotic bacteria under control. To my knowledge, research on this topic in Coleoptera has been mostly focused on the cereal weevil *Sitophilus* sp. and its obligate endosymbiont *Sodalis pierontonius.* This beetle tightly modulates its symbiotic titer possibly by apoptotic and autophagocytic mechanisms as

well as the host-derived anti-microbial peptide (AMP) coleoptecirin A [152]. Studies on *Drosophila* flies revealed that AMPs produced by the Toll (acting mostly against gram-positives) and Imd (acting mostly against gram-negatives) pathways are major prevention methods of symbiotic bacteria growing off-limits [153]. Current data suggest that the resident microbiota may be able to stimulate production of host AMPs in response to pathogenic threats, thus avoiding at the same time a disease scenario and being displaced by invading microorganisms [154], [155]. Other important host immune effectors are the reactive oxygen species (ROS) produced by the dual oxidases (DUOXs) [156]. In *Drosophila*, DUOXs are specifically activated upon pathogen infection [157] and silencing of these enzymes leads to an increase in gut bacterial populations of *Anopheles* mosquitoes [158]. In some cases the insect may attenuate the immune response towards a beneficial bacterium [152], [159] or regulate the population of a particular symbiont in so extremely fine-tuned way that only the symbiotic strain and not the environmental one is inhibited by host AMPs [160]. Outside the immune system, enteroendocrine cells and neurons of *Drosophila* flies might create a host-microbe feedback that contributes to community regulation as well [161]. Parallel to the mentioned host-derived mechanisms, some microbial traits may also influence the symbiotic milieu. These may be inherent to a particular bacterium, such as pathogen-like secretion systems that help in evading host immunity [152]; also, they may result from mutualistic or deleterious microbe-microbe interactions, as is the case of *Asaia* sp. and *Acinetobacter* sp. that enhance each other's growth in the digestive tract of *Aedes albopicus* [162] or the negative interaction occurring between *Serratia* sp. and other symbionts of the gut of the locust *Schistocerca gregaria* [163]. The secretion of bacteriocins may also disrupt the membrane of competing bacteria [144]. Altogether, the main purpose of such display of regulatory strategies is to control and adjust the symbiotic population to the particular nutritional needs of each stage of host development.

It is plausible that, in *M. hippocastani*, immunity pathways homolog to those of *Drosophila* flies play a role in orchestrating the observed shifts in the composition of the hindgut wall community [152]. Secretion of ROS might take place as well, as suggested by the numerous differentially expressed catalases and superoxide dismutases by hindgut wall Firmicutes and a differentially expressed insect DUOX in the pocket (Unpublished results). Although speculative, it is possible that the sequential variation of the hindgut wall symbiotic community is in first instance conditioned by the mechanism of transgenerational transmission of the symbionts. Previous

experiments involving Fluorescent In Situ Hybridization (FISH) with the general bacterial probe EUB-338 revealed a thick bacterial layer covering the shell of recently laid eggs of *M. hippocastani*, pointing towards a symbiont transmission by the egg smearing method [3], [164], (Arias-Cordero et al., unpublished results). Since the egg is laid underground, aerobic or microaerophilic soil bacteria might outgrow the anaerobic symbionts overlaying the eggshell and, after ingestion by the newborn larva, these environmental bacteria might attach and successfully thrive on the hindgut wall during early host developmental stages (until second instar) before it becomes too anaerobic. This hypothesis is supported by the ubiquity of hindgut wall dominant families in second instar larvae (Pseudomonadaceae and Caulobacteraceae) among native soil microbes [151], [165], [166]. Alternatively, these families might belong to the gut symbiotic community, albeit in very low amounts in late host development (third instar larvae and adults), and be enriched on the eggshell due to the deleterious effects of oxygen on their anaerobic competitors. In any case, the observed dynamics are likely to follow nutritional purposes. The Pseudomonadaceae family, most prominent in the second instar larvae, has been reported to degrade uric acid in insects and snails [100], [167]. Thus, they might contribute to break down *M. hippocastani*'s nitrogenous waste and provide the necessary ammonia for *de novo* amino acid synthesis by other bacteria, possibly *Burkholderia* sp. (Article II). Working in synergy, Pseudomonadaceae members and *Burkholderia* sp. might constitute a valuable amino acid source in a stage of host development in which growth, and therefore protein synthesis, is crucial. Both Pseudomonadaceae and Caulobacteraceae families include cellulolytic genera [166], [168] but since Caulobacteraceae are regarded as strict aerobes and Pseudomonadaceae are non-fermenters [151], it is likely that the amount of symbiont-produced acetate, a valuable carbon and energy source for the host [87], [142], is negligible in second instar larvae. This may limit the energetic yield of ingested plant polysaccharides. In contrast, the increase of fermentative and acetogenic bacteria such as Clostridiales or Bacteroidales [169] in third instar larvae may ensure an abundant supply of fatty acids that may be stored in the insect's fat body in preparation for the forthcoming and energetically costly metamorphosis phase. In adults, a fermentative symbiotic community similar to third instar larvae might contribute in providing the required energy for flight [170].

5.5 Bacterial community and possible biological role of the pockets

As mentioned in the introduction, in the mid-50s Wildbolz briefly reported the existence of unusual raceme-shaped structures attached at the distal end of the hindgut of *M. melolontha*, a close species to *M. hippocastani*. Wildbolz highlighted that these structures were filled with a "dark mass" and their intima layer seemed "thickened". Such formations were also present in rhizophagous larvae of the genera *Anomala*, but absent in *Oryctes* and *Phyllognathus*, whose grub-like larvae live and feed on decaying wood [115]. This suggest a relation of these enigmatic structures with a rhizophagous diet and/or underground lifestyle; however, as Wildbolz himself acknowledged, their precise function was then unknown, and so remained until today.

We surveyed again these formations, regarded from now on as 'pockets', using modern microscopy and DNA and RNA sequencing techniques. Our analysis made possible to unmask a taxonomic dissimilarity between the hindgut wall- and pocket bacterial communities. While in second instar larvae the hindgut wall was colonized mostly by the families Pseudomonadaceae and Caulobacteraceae (see section 4.3), the most numerous families in the pockets were Micrococcaceae and Alcaligenaceae; within the latter, 85% of sequences belonged to the genus *Achromobacter*, rendering it the most ubiquitous indentified genus in the pockets (Article III). Later, RNA sequencing (RNAseq) analysis revealed that the vast majority of pocket transcripts aligned to *Achromobacter* sp. (Unpublished results), suggesting that the Micrococcaceae may be inactive or end up displaced by Alcaligenaceae. Moreover, Transmission Electron Microscopty (TEM) unveiled that the pockets were lined with a highly dense bacterial population and revealed that many members possessed intracellular granules visually resembling poly-β-hydroxybutyrate (PHB). The composition of such droplets was confirmed by gas chromatography of pocket-isolated *Achromobacter* sp. and Raman microspectroscopy of whole pocket tissue (Article III). PHB is a type of polyhydroxyalkanoate accumulated by many bacterial and archaeal species upon high environmental carbon concentrations [171], which is a logical condition of the gut of an insect thriving in a carbon-rich diet such as roots [135]. Moreover, PHB accumulation may be decisive for the succesful symbiotic colonization of the pockets, in the same way as it is for the colonzation of the midgut crypts of the bean bug *Riptortus pedestris* by its burkholderial symbiont [48]. The key might be the broad protection against stress that PHB accumulation confers, including osmotic, oxidative and nutritional,

among others [48] [172], possibly due to that PHB constitutes a reliable nutrient source when the uptake from the environment is hampered by the host-induced unfavorable conditions [173]. PHB accumulation is rare among verically-transmitted, well-adapted gut symbionts, as they do not have to face the unexpected and sometimes abrupt nutritional shortages inherents of a free lifestyle [48],[174]. The fact of pocket-isolated *Achromobacter* showing this unusual trait and the close alignment of its 16S rDNA gene sequence with root and soil isolates (Article III) suggest that this symbiont comes from the larval surroundings and it is possibly ingested *de novo* each generation.

The most comparable insect-symbiont system to *Melolontha-Achromobacter* is *Riptotus-Burkholderia*. Both microbes belong to the β-proteobacteria class, are commonly rhizosphere-associated, present PHB accumulation and colonize a specific niche within the host digestive tract [175]. However, since differences are observed as well, functional parallelisms must be established with caution. Firstly, the *Riptortus* crypts are located in the distal section of the midgut, while the *Melolontha* pockets are found in the distal section of the hindgut. This remote location renders unlikely a detoxification role analogous to that of the crypts [104], as ingested toxins would have travelled and affected the whole digestive tract before reaching the detoxification site. A role in nitrogen recycling suggested by similar location and symbiont taxonomy to the pouch-shaped organ of *Tetraponera* ants [68] appears not to be specific of the pockets, as no differential expression of ureases or uricases was detected. Nevertheless, expression of the glutamine synthetase pathway implies that pocket bacteria may synthetize amino acids using hindgut-produced ammonia, although this process may be carried out by hindgut wall symbionts as well. No genetic evidence of production of vitamins and cofactors that might benefit the host was detected in the pockets; however, they might contribute to the absorption of micronutrients such as sulphur (Unpublished results). In any case, the main function of these enigmatic structures, as pointed out by outcome of the RNAseq, might not be nutritional but immunological. A vast array of host immune genes related mostly to the melanization response [176] and cuticular proteins [177], [178] were expressed in pocket tissue, suggesting activation of the immune system. It has been reported that in the *Riptortus-Burkholderia* system, presence of *Burkholderia* in the crypts stimulates host immunity and causes a more intense secretion of AMPs in response to pathogenic challenge [179]. Such scenario makes sense in the context of *M. hippocastani* physiology. The larvae may require a

strong immune system in order to survive up to 4 years in a bacteria-rich environment such as the rhizosphere and to control their highly diverse gut microbiome. Contrary, the adults may not need any immune-stimulation to successfully go through their ephemeral lifespan of only 4-6 weeks and therefore lack pockets. Similar immunity decoupling is reported in *Anopheles* mosquitoes, whose immunity rapidly declines after metamorphosis [180], and in *Drosophila* flies, in which the expression pattern of the antifungal peptide drosomycin varies from larvae to adult [181]. Additionally, RNAseq unveiled an increased expression in the pocket of insect Osiris protein-family, arylphorin transcripts and a highly expressed insulin-like growth factor. The specific role of the Osiris family is unknown, but the fact that they peak at determined developmental time points (embryo, second instar larvae and pupae) and the simultaneous expression of arylphorins and an insulin-like growth factor might indicate a role of the pockets in host development. In the *Riptortus-Burkholderia* system it has been reported that presence of the symbiont increases the expression of hexamerins (arylphorins are a subfamily of hexamerins) [182]. In *Aedes* mosquitoes, environmentally-acquired gram-negative symbionts provoke a decrease in oxygen level in the gut that triggers molting, presumably by affecting the signaling by insulin-like peptides and 20-hydroxyecdysone [183]. Interestingly, three glucose dehydrogenases transcripts were highly expressed in pockets, which may be correlated to the presence of 20-hydroxyecdysone in the tissue [184]. Further hints pointing towards occurrence of 20-hydroxyecdysone are the highly expressed arylphorins, which may function as ecdysteroid hormone carriers [185] as well as the variable and apparently random coloration (black or white) of the pockets depending on individual, which might be due the inhibition of melanotic pigmentation by 20-hydroxyecdysone [186]. Also an insulin-like growth factor binding protein was expressed uniquely in the pockets. Altogether, the expression of these transcripts suggests an involvement of the pockets in the regulation of host molting process.

5.6 Comparison of the symbiotic communities of *Spodoptera littoralis* and *Melolontha hippocastani*

Considering the striking differences between the diets of *M. hippocastani* and *S. littoralis* larvae, it is plausible that this factor greatly influences the anatomy and the physiochemical conditions of their guts and, by extension, the composition of the symbiotic communities [8], [187]. The straight, simple shaped digestive tube of *S. littoralis* larvae favors a rapid transit of the food

bolus, while in *M. hippocastani* it is slowed down by the proctodeal dilatation and the hindgut chamber [115]. Moreover, *S. littoralis* maintains a highly alkaline midgut pH in order to increase nutrient absorption from plant tissue [188]. Both fast passage of food and high gut pH create harsh environmental conditions that limit the diversity of symbiotic bacteria able to colonize the lepidopteran digestive tract [120], [189]. In the scarabaeid larva, a milder gut pH [16] and the protrusion lobes of the hindgut cuticle that provide bacterial anchorage (Arias-Cordero, unpublished results) facilitate the colonization by a wider diversity of bacterial symbionts. The slow food passage throughout *M. hippocastani*'s gut suggest a dependence on its symbionts for the digestion recalcitrant polysaccharides and the presence of specialized structures for symbiont housing (the pockets) in the larval hindgut further supports the idea of close relationship between the scarabaeid and its gut community [134]. Similar structures are not common among lepidopterans [190]. Taken together, these observations suggest that *S. littoralis* might not depend on their symbionts at the same extent than *M. hippocastani*.

Another force conditioning the composition of the gut bacterial community is the oxygen regime. The gut wall is usually populated by scavengers that consume oxygen and contribute in the creation of an anoxic environment in the lumen [16]. Furthermore, the enlarged hindgut chamber of the scarabaeid larvae further limits oxygen diffusion towards the central part of the lumen. Anoxic conditions are likely to occur in the gut of both insects studied in this thesis, although in *S. littoralis* the gut might be more aerobic as compared to *M. hippocastani*. In the lepidopteran anaerobic bacteria were mostly found in the lumen [189] while in the coleopteran anaerobic bacteria end up profusely colonizing the hindgut wall in late-instar larvae (Article III). Oxidative stress may be also generated by the secretion of reactive oxygen species (ROS) used by the host to control the microbial population [156]. Leaf phenolic compounds are reported to have antimicrobial properties and influence on the gut microbiome [109],[191]. They may be related to the lower bacterial diversity of *S. littoralis* larvae gut as compared to *M. hippocastani*, as well as to the observed decrease of bacterial diversity in *M. hippocastani* adult gut (folivore) as compared to larvae (rhizophagous) (Article III). Finally, the redox potential of the digestive tract may also shape the bacterial community; nevertheless, this is not likely to be a differentiating trait between the two insects under study, as they show similar redox profiles, being more oxidative in the midgut [13], [188].

Despite the marked differences in diet and gut structure and physiology, in both insects a core community well differentiated from the indigenous bacteria of the food is consistent through generations [114], [189]. It is likely that the insect actively modulates the composition of the core microbiome in order to meet its nutritional needs. This is observed in *S. littoralis*, whose symbiotic community varies when changing the host plant [189]. Given its consistency across host's generations, it is probable that these bacteria are transmitted from mother to offspring possibly by a mechanism involving an inoculum carried via the eggs (Arias-Cordero, unpublished results), as it happens in *Manduca sexta* and *Melolontha melolontha* [46], [192].

6. Future perspectives

This thesis provided insight into the key microbial taxa processing cellulose and recycling nitrogenous waste within the highly diverse gut bacterial community of *M. hippocastani*. Since the existence of obligate symbionts is not reported for this insect the isolation and culturing of the Illumina-SIP highlighted microbes should be feasible. This opens the door not only to functional in vitro experiments, but also to the sequencing of their genome, which could be used as a reference for future RNAseq experiments involving not only the hindgut wall but also the lumen. It is also of utmost interest to unravel the host regulatory mechanisms that may be behind the shift in metabolically active bacteria between larvae and adult stages showed in Article I as well as the variation in hindgut wall community composition across larval stages showed in Article III. A proteomic approach would be useful to address this question and might lead to the discovery of new insect AMPs. However, research in *M. hippocastani* suffers an important drawback, and that is the impossibility to rear consecutive generations of this insect in the laboratory. This deprives us of the obtainment of aposymbiotic insects that could be used to determine the degree of dependence of the insect to its symbionts or the mechanisms that orchestrate the bacterial colonization of the digestive tract. Also, breeding the insect in the laboratory would render possible, although challenging due to its prolonged life cycle, to obtain mutant lines usable for genetic studies and ultimately to compose a genetic toolbox similar to that available for *Drosophila* flies. Thus, it is imperative that part of the future work in *M. hippocastani* is devoted to the elaboration of a rearing protocol.

This thesis also served to provide the first insight on the composition of the bacterial community inhabiting the pockets and on their function in the context of insect physiology. The differential gene expression analysis presented in Unpublished results suggested that they could be involved in immune stimulation and/or host development regulation. However, those are only hypotheses and their confirmation is the logical forthcoming step. To accomplish this, it may be useful to take advantage of the radical taxonomic difference between the active pocket and hindgut wall communities (gram-negative versus gram-positive). By means of gram-negative targeted antibiotics it may be possible to selectively suppress the pocket bacteria and to test whether this has an effect on host fitness, development and/or survival. Additionally, a more comprehensive RNAseq experiment involving not only second instar- but also first- and third instar larvae might

help to further clarify the function of the pockets and to unveil the genetic mechanisms behind the selective symbiotic colonization of these niches. Since we did not have reference genomes, neither for *M. hippocastani* nor for any of its bacterial symbionts, a read length of 250 bp was used as this may help in *de novo* assembling the transcriptome. Nevertheless, future experiments should be performed with a read length of 100 bp for a more comprehensive picture of the gene expression.

Finally, the genome sequence obtained from *E. mundtii* isolated from the gut of the lepidopteran pest *S. littoralis* provided an array of putative roles of this bacterium within the gut. However, some of the detected genes may be expressed only under certain conditions or not be expressed at all. Therefore, a RNA-based approach is necessary in order to more precisely determine the function of *E. mundtii*. Such experiment is currently in progress: by GFP-labeling isolated *E. mundtii* and feeding it back to the larvae, the fluorescent bacterium can be selectively re-isolated using a Fluorescence Activated Cell Sorting device (FACS), allowing RNA extraction and sequencing without interference of host or other symbionts RNA. Since there is an established laboratory rearing protocol for *S. littoralis*, the conditions under which the symbiont is isolated can be easily controlled, providing uncountable possibilities for functional genomics. Moreover, by using a fluorescence microscope it is possible to directly visualize the GFP-labeled *E. mundtii* within the gut and to study the mechanisms behind its transfer across metamorphosis, from host larval to adult stages.

7. Summary

7.1 English

Insects are the most widespread animal group of the planet and symbiotic association with microorganisms, especially bacteria, is one of the keys of their success. Through this association, the insect benefits from the virtually unlimited metabolic capabilities of the prokaryotes and is able to thrive in hostile ecological niches. In this thesis, the symbiotic communities of two insects were functionally approached: the specialist *Melolontha. hippocastani* and the generalist *Spodoptera littoralis*. The main focus was put on the former due to its particular life cycle comprising two well differentiated stages (a root-feeding larval stage and a leaf-feeding adult stage) and the surprising stability of its core microbiome. Therefore, it was expected to find some variation on the active fraction of the community coupled with the transition from larval to adult stage (Article I). Parallel to this, the genome of *Enterococcus mundtii*, a prominent symbiotic bacterium of the gut of *S. littoralis* was sequenced, shedding some light on the role of this bacterium within the lepidopteran gut and providing a valuable reference genome for future genomic studies (Article II). Moreover, since scarabaeids beetles such as *M. hippocastani* rely on their hindgut fermentation chamber for symbiotic digestion of recalcitrant polysaccharides, the dynamics of the hindgut wall microbial community was investigated at different life stages in order to unveil potential stage-dependent variations. Also, the symbiotic population of specialized bacterial niches of the larval hindgut (the pockets) was characterized for the first time (Article III). Comparative analysis of the metatranscriptome of the hindgut wall and the pockets allowed to draw inferences on the function of the latter (Unpublished results).

Function and structure of the symbiotic community of *Melolontha hippocastani*

M. hippocastani possess a stable core bacterial community that does not significantly vary despite the radical change of habitat that the insect undergoes after metamorphosis. Therefore, we hypothesized that a taxonomic shift in metabolically active bacteria must result in adaptation to the new diet. A Stable Isotope Probing - Illumina sequencing experiment (Illumina-SIP) was designed in order to test that. Cellulose and urea were used as trophic links, as the former is the main dietary compound of a herbivorous insect and the latter constitutes, together with uric acid, the nitrogenous waste pool of insects. The results pointed towards the bacterial families

Lachnospiraceae and Enterococcaceae in larvae and Enterobacteriaceae in adults as involved in cellulose processing, confirming the occurrence of the hypothesized taxonomic shift. Labeling with ^{15}N urea revealed that this shift extends to nitrogen recycling bacteria as well, as the genus *Burkholderia* was isotopically labeled in larvae and the genus *Parabacteroides* in adults (Article I). These shifts may be consequence to the differences in carbon and nitrogen needs that may exist between larval and adult *M. hippocastani*. Despite the stable core community, local variations in taxonomic composition are observed as well. 454-pyrosequencing of the hindgut wall revealed that in second instar larvae its symbiotic milieu was composed mainly by the Caulobacteraceae and Pseudomonadaceae families, while in third instar the dominant families were Porphyromonadaceae and Bacteroidales-related. The hindgut wall community of adults was fairly similar to third instar larvae with a significant increase of the Enterobacteriaceae family (Article III). The observed dynamics may be regulated by the host according to its dietary requirements, as suggested, for example, by the increase of fermentative bacterial families in third instar larvae, when the amount of ingested food and therefore fermentable plant polysaccharides is bigger.

Role of *Enterococcus mundtii* within the gut of *Spodoptera littoralis* suggested by its genome sequence

Previous studies determined that the gut bacterial community of *S. littoralis*, as of lepidopteran larvae in general, is not very rich, possibly due to the harsh physiochemical conditions and the rapid food passage. As *E. mundtii* had been targeted as a metabolically active symbiont in the gut of *S. littoralis* by ^{13}C-glucose Stable Isotope Probing, isolation of this bacterium and sequencing of its genome was imperative in order the get insight on its physiological function and to provide a reference genome for future gene expression analyses (Article II). *E. mundtii* genome presented an elevated percentage of coding capacity (12%) devoted to carbohydrate uptake and processing and the predicted pathways suggested that it might be capable of producing fermentation metabolites such as acetate or formate. Moreover, it was unveiled a high number of genes potentially involved in assuring the persistence of the bacterium in the gut, such as biofilm formation or bacteriocin production. Altogether, the genome of *E. mundtii* suggests that this symbiont plays a crucial role in the digestion of host diet.

Bacterial community and potential role of *Melolontha hippocastani* hindgut pockets

Previous observations of *M. hippocastani* hindgut spotted specialized organs of unknown function (pockets). A survey of these structures was conducted using modern microscopy and sequencing techniques, revealing that they were filled with a highly dense bacterial population accumulating intracellular white-looking droplets. 454 pyrosequencing allowed identifying these bacteria as *Achromobacter* sp. and Micrococcaceae-related, although later RNA sequencing (RNAseq) analyses showed that the Micrococcaceae family was barely expressing any gene. *Achromobacter* sp. was isolated from the pockets and Gas Chromatograhy – Mass Spectrometry analysis revealed that this bacterium accumulated poly-β-hydroxybutyrate (PHB) *in vitro*. Presence of PHB was also detected in whole pocket tissue by Raman microspectroscopy. The exact role of this polymer is yet unknown but it is plausible that its presence is crucial for the successful symbiotic colonization of the pockets. Further research is needed to elucidate the function of the pockets in the context of insect physiology, although some hypothesis can be inferred based on the RNAseq outcome, which pointed towards an involvement of these enigmatic structures in regulation of host development and/or immunity stimulation.

7.2 German

Insekten stellen die vielfältigste Klasse der Gliederfüßer sowie die artenreichste Klasse der Tiere weltweit dar. Der Erfolg diverser Insektenarten wird unter anderem durch symbiotische Verbindungen mit Mikroorganismen wie unter anderem Bakterien ermöglicht. Mithilfe dieser Assoziation ist das Insekt in der Lage, von den unerschöpflichen metabolischen Ressourcen des Prokaryoten zu profitieren, um auch in nicht optimalen ökologischen Nischen überleben zu können. In dieser Arbeit wird nachfolgend die symbiotische Beziehung zwischen dem Spezialisten *Melolontha hippocastani* und dem Generalisten *Spodoptera littoralis* erläutert. Der Spezialist *M. hippocastani* weist einen spezifischen Lebenszyklus auf, der eine larvale Phase mit Wurzeln sowie einer adulten Phase mit Blattmaterial als Nahrungsquelle umfaßt. Durch diesen distinkten Lebenszyklus einhergehend mit einem unerwartet stabilen Mikrobiom liegt der Schwerpunkt dieser Arbeit auf dem Herbivoren *M. hippocastani*. Aufgrund der Stabilität des Mikrobiomes in *M. hippocastani* konnte eine Veränderung der Zusammensetzung der mikrobiellen Artengemeinschaft während des Übergangs von dem larvalen in das adulte Stadium aufgezeigt werden (Artikel I). Parallel dazu, wurde das Genom des am häufigsten vorkommenden Darmbakteriums in *S. littoralis*, *Enterococcus mundtii* sequenziert. Die aus der

Sequenzierung gewonnenen Informationen zeigen tiefergehend die Relevanz dieses Bakteriums innerhalb des Verdauungstraktes von *S. littoralis* auf und können weiterführend als Referenz für genomische Studien verwendet werden (Artikel II). Außerdem, in Vertretern der Familie der Blatthornkäfer (Scarabeidae) wie z.B. *M. hippocastani* findet in der Hinterdarms Fermentationkammer der symbiotische Verdau von abbauresistenten Polysacchariden statt. Um die Dynamik der Mikrobiota -Zusammensetzung der Dickdarmwand zu untersuchen, wurde diese in unterschiedlichen Phasen des Lebenszyklus charakterisiert. Zusätzlich wurden die „Taschen " des larvalen Hinterdarms im Hinblick auf bakterielle Nischen spezialisierte symbiotische Populationen erstmalig beschrieben (Artikel III). Vergleichende Analysen des Metatranskriptoms der Hinterdarmwand und der „Taschen" ermöglichen Rückschlüsse auf die Funktion der Letzteren (nicht veröffentlichte Ergebnisse).

Struktur und Funktionen der symbiotischen Gemeinschaft in *Melolontha hippocastani*

M. hippocastani besitzt eine stabile bakterielle Kerngemeinschaft, die trotz der radikalen Veränderung des Habitats während der Metamorphose des Insekts nicht variiert. Aufgrund dieser Beobachtung entstand die Hypothese, dass eine taxonomische Verschiebung der metabolisch aktiven Bakterien eine Anpassung an die neue Ernährung darstellt. Zur Verifizierung dieser Annahme wurde die Methode des „Stabile Isotopensondierung – Illumina Sequenzierung" angewandt. Zellulose stellt den Hauptbestandteil der Nahrung herbivorer Insekten dar; Urea in Kombination mit Harnsäure macht den stickstoffhaltigen Verdauungsabfall aus. Diese zwei Komponenten wurden folglich als trophische Verbindungen verwendet. Es konnte erfolgreich gezeigt werden, dass Vertreter der Familien Lachnospiraceae und Enterococcaceae in den Larven, sowie Enterobacteriaceae in den Adulten eine Rolle in der Verarbeitung von Zellulose spielen. Dies bestätigt die zu Beginn angenommene taxonomische Verschiebung. ^{15}N Urea Sondierung zeigte desweiteren, dass diese Verschiebung auch Stickstoff-wiederverwertende Bakterien umfaßt, da unter anderem *Burkholderia* in den Larven, sowie *Parabacteroides* in den Adulten isotopisch markiert waren (Artikel I). Diese Verschiebungen könnten darauf beruhen, dass *M. hippocastani* je nach Lebensabschnitt unterschiedliche Mengen an Kohlenstoff und Stickstoff benötigt. Abgesehen von der stabilen bakteriellen Kerngemeinschaft konnten lokale Veränderungen in der taxonomischen Zusammensetzung beobachtet werden. 454-Pyrosequenzierung der Hinterdarmwand konnte zeigen, dass das symbiotische Milieu von

Larven im zweiten Stadium hauptsächlich aus Vertretern der Caulobacteraceae und Pseudomonaceae Familien besteht. Im dritten Stadium stellten Porphyromonadaceae und Verwandte der Familie der Bacteroidales den größten Teil der bakteriellen Gemeinschaft dar. Verglichen mit der bakteriellen Zusammensetzung des dritten Larvenstadiums konnte in den Adulten ein signifikanter Anstieg der Häufigkeit der Enterobateriaceae festgestellt werden (Artikel III). Die hier beschriebene Dynamik in der Zusammensetzung der mikrobiellen Gemeinschaft könnte angepasst an die Nahrungsquelle des Wirts erfolgen. Wird beispielsweise die Menge an Futter und somit zu verdauenden Polysacchariden erhöht, wird parallel dazu die Anzahl an fermentierenden Bakterienfamilien hochreguliert (s. Larve im dritten Stadium).

Die Rolle von *Enterococcus mundtii* innerhalb des Darms von *Spodoptera littoralis* im Kontext der Genomsequenz

Vorangehende Studien zeigten, dass die bakterielle Zusammensetzung des Darms von *S. littoralis*, sowie in Larven der Art Lepidoptera im Allgemeinen, vergleichsweise schwach ausgeprägt ist. Eine mögliche Erklärung dessen könnten die rauen vorherrschenden Bedingungen sowie die schnelle Passage der Nahrung durch den Darm sein. Im Zuge der vorangegangenen Experimente mittels ^{13}C-glucose Stabile Isotopensondierung konnte *E. mundtii* als metabolisch aktiver Symbiont im Darm von *S. littoralis* gezeichnet. Isolierung und Genomsequenzierung solches Bakteriums wurde durchgeführt (Artikel II). Das sequenzierte Genom von *E. mundtii* wies unter anderem eine erhöhte kodierende Kapazität (12%) für die Aufnahme bzw. Verarbeitung von Kohlenhydraten auf. Der prognostizierte metabolische Wege deuteten darauf hin, dass *E. mundtii* in der Lage wäre, Fermentationsmetabolite wie Acetat oder Formiat zu produzieren. Darüber hinaus wurde eine große Anzahl von Genen enthüllt (wie z.B Biofilmbildung oder Bakteriocinproduktion) die potenziell dazu beitragen, die Persistenz des Bakteriums im Darm zu gewährleisten. Zusammenfassend legt das Genom von *E. mundtii* nahe, dass dieser Symbiont eine entscheidende Rolle bei der Verdauung der Ernährung des Wirts spielt.

Bakterielle Gemeinschaft und mögliche Rolle von *Melolontha hippocastani* Hinterdarm Taschen

Frühere Beobachtungen von *M. hippocastani* Hinterdarm haben spezialisierte Organe unbekannter Funktion (Taschen) entdeckt. Eine Untersuchung dieser Strukturen wurde unter Verwendung moderner Mikroskopie und Sequenzierungstechniken durchgeführt, die enthüllte, dass sie mit einer hochdichten Bakterienpopulation gefüllt waren, die intrazelluläre, weiß aussehende Tröpfchen akkumulierte. Mit 454-Pyrosequenzierung konnten diese Bakterien als *Achromobacter* sp. und Micrococcaceae verwandt identifiziert werden. Spätere RNA-Sequenzierung (RNAseq) Analyse zeigte jedoch, dass die Familie der Micrococcaceae kaum Gene exprimierte. *Achromobacter* sp. wurde aus den Taschen isoliert und Gaschromatograhie – Massenspektrometrie Analyse zeigte, dass dieses Bakterium Poly-β-Hydroxybutyrat (PHB) *in vitro* akkumulierte. Das Vorhandensein von PHB wurde auch im gesamten Taschengewebe durch Raman-Mikrospektroskopie nachgewiesen. Die genaue Rolle dieses Polymers ist noch unbekannt, aber es ist plausibel, dass seine Anwesenheit für die erfolgreiche symbiotische Kolonisierung der Taschen entscheidend ist. Weitere Untersuchungen sind notwendig, um die Funktion der Taschen im Zusammenhang mit der Insektenphysiologie aufzuklären, obwohl einige Hypothesen basierend auf dem RNAseq-Ergebnis abgeleitet werden können, was auf eine Beteiligung dieser rätselhaften Strukturen bei der Regulation der Wirtsentwicklung und / oder Immunstimulation hinweist.

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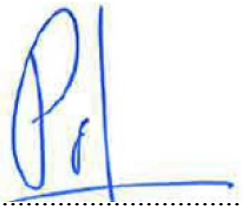
9. Eigenständigkeiterklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, dass ich die vorliegende Dissertation eingenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitel genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingericht. Weiterhin wurde keine ähnliche oder andere Abhandlung als Dissertation anderswo eingericht.



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Pol Alonso Pernas

10. Acknowledgments

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